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II. Spring diatom bloom density varied inversely with the preceding winter's blue-green population density. Diatom blooms, when they occurred, ended when available silica was depleted. Generalized *in situ* and *in vitro* inhibition of diatoms by blue-greens. After separation and concentration via ether extraction or ultrafiltration active substances were returned to growth media. Preliminary evidence suggests that inhibition involves interference with silica availability.

III. The feasibility of biological programming of bloom sequence in eutrophied lakes is considered.

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July 1976

ALGAL METABOLITE INFLUENCE ON BLOOM SEQUENCE IN EUTROPHIED FRESHWATER PONDS

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ABSTRACT

I. Bloom sequence in Linsley Pond, Connecticut, was monitored for three years. Bloom dominant algae were isolated in culture, heat-labile, bio-active substances in cell-free filtrates of these cultures were tested against each of the dominants. Enhancing, or neutral, effects on successors; and inhibiting, or neutral, effects on predecessors were consistently observed. Lake waters exhibited parallel effects. Additionally, inhibition patterns suited differences in year-to-year patterns of in situ blooms. This widespread correlation of in situ events with in vitro phenomena indicates that extracellular products of bloom dominant algae are significant in bloom sequence determination in eutrophied fresh waters.

II. Spring diatom bloom density varied inversely with the preceding winter's blue-green population density. Diatom blooms, when they occurred, ended when available silica was depleted. Generalized in situ and in vitro inhibition of diatoms by blue-greens was traced to heat-labile, dialysable products of blue-greens. After separation and concentration via ether extraction or ultrafiltration active substances were returned to growth media. Preliminary evidence suggests that inhibition involves interference with silica availability.

III. The feasibility of biological programming of bloom sequence in eutrophied lakes is considered.

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SECTION I

CONCLUSIONS

GENERAL

1. While not the sole parameter of control, the extracellular metabolites of planktonic bloom dominant algae play a most significant role in the determination of bloom sequence in a eutrophied freshwater pond.

2. Certain extracellular metabolites of planktonic blue-green algae substantially inhibit the growth of planktonic diatoms in culture and in the natural sequence in Linsley Pond.

3. Preliminary tests indicate that the inhibition of diatom growth by blue-green algal metabolites may be widespread in freshwater lakes.

4. When the elimination of excessive nutrient inflow is not practical, biological management, or programming, of blooms in eutrophied lakes should be attempted. An hypothetical plan is offered to modify the unsatisfactory conditions in one lake which would cost approximately $500 per annum while providing a more satisfactory lake from both aesthetic and food chain points of view; Section IV.

SPECIFIC

1. Bloom sequence, cell-free filtrates of bloom dominant blue-green algae, and freshly collected lake waters provided in vitro and in situ examples of algal allelopathic effects on blue-greens, diatoms, flagellates, and green algae.

2. Cell-free filtrates of eight planktonic blue-greens which dominated in Linsley (three year sequence) all produced growth inhibiting effects on the 30 plus varieties of planktonic Linsley diatoms tested, and on the non-Linsley diatoms tested.

3. Two non-Linsley blue-greens produced inhibitory effects on most, but not all, of the Linsley and non-Linsley diatoms tested.

4. The presence of bacteria in producer and, or, assay cultures diminished, but did not eliminate, the effects noted above.

5. The substance responsible for diatom inhibition in cell-free filtrates of the blue-green Anabaena sp. (538) can be separated and concentrated by ether extraction or by ultrafiltration without loss of activity.

6. This diatom inhibiting substance is heat-labile; dialysable; and can be separated into the following three fractions:

1) Inhibitor #1
Color: Peach-pink (Winkler pink)
MW: Less than 12,000 and greater than 10,000
Size: 20-50 Å (as indicated by dialysis and Amicon ultrafiltration)
Other: Oily to touch

2) Enhancer #1
Color: Vivid mid-yellow
MW: Less than 10,000 and greater than 1,000
Size: 10-20 Å
Other: Adheres to filter, therefore may be a steroid. Is masked by Inhibitor #1 at full strength, but not at dilution.

3) Inhibitor #2
Color: None
MW: Less than 500
Size: Less than 10 Å
Other: May be an artifact of bioassay procedures, especially of autoclaving.

7. The addition of moderately high quantities of silica overcomes in part, or totally, the heat-labile inhibitory effects of blue-greens on diatoms.

8. Densities of spring diatom blooms in Linsley Pond are inversely proportional to those of winter blue-green blooms.

9. Waters collected at the end of spring diatom blooms in Linsley Pond support very low diatom growth; diatom growth can be restored by the addition of silica.

10. Blue-green bloom populations develop in Linsley Pond after the spring diatom bloom wanes.

11. Zooplankton populations in Linsley vary inversely with blue-green populations.

SECTION II

RECOMMENDATIONS

1. The biological management of bloom sequence, as a means to ameliorate conditions of eutrophication when nutrient elimination is not practical, should be thoroughly explored. Demonstration projects in severely eutrophied small lakes should be attempted. While Linsley is a likely target, and an hypothetical program for such control is outlined in this report, Section IV, the very special value of Linsley to the scientific community may make any form of intervention undesirable. The suggested program could be readily adapted to other lakes in similar condition if suitable data concerning the bloom sequence of those lakes were available. While the approach would be similar, specific management techniques must be tailored to the lake in question.

2. Further work should be undertaken to determine how general the blue-green inhibition of diatom growth is. This would allow the application of management techniques to incorporate additional controls for undesirable blooms.

3. Since preliminary study suggests that substances in the cell-free filtrates of blue-green cultures interfere with *in vitro* growth of the crustacean, *Moina macrocopa*, and since zooplankton populations in many freshwater lakes have been found to vary inversely with blue-green populations, the possibility that blue-green metabolites may effect the next trophic level should be explored carefully. The rapid disappearance of zooplankton during blue-green blooms has been tied to food preference and digestability problems in prior studies. But no indication has heretofore been presented that there are allelopathic, extracellular metabolite, effects.

SECTION III

CORRELATIONS OF IN SITU—IN VITRO EVENTS AND THEIR IMPLICATIONS

III:I CORRELATION OF BLOOM SEQUENCE WITH BIOLOGICAL ACTIVITY IN FILTRATES OF DOMINANT BLUE-GREEN ALGAE

The correlation between bloom sequence and heat-labile filtrate effects presents a consistent picture of bloom dominant blue-greens producing 1) inhibiting or neutral (-, 0) effects on predecessors, and 2) enhancing or neutral (+, 0) effects on successors (Table 1). Therefore, the extracellular metabolites in filtrates show a capacity to improve the competitive position of the organism which will dominate in an impending bloom by accelerating the elimination of the predecessor and by increasing the growth potential of the new dominant. In addition filtrate studies suggest an exclusion capacity; *i.e.*, the metabolic products of blue-green bloom dominants appear to exclude from the sequence in eutrophied waters, seasonal blooms which commonly occur in oligotrophic and mesotrophic waters. Naturally, such biological activity cannot be considered the only basis for a specific organism dominating a bloom, but it does substantially limit the number of organisms likely to achieve such dominance. This limit, when correlated with the many physical and chemical parameters of growth control, would provide a very few, if not one, possible dominant for any given place in the bloom sequence.

Although the next organism studied may prove to be the first exception, there are presently no indications that *in vitro* metabolite activity works in opposition to the *in situ* bloom sequence. That is, none of the filtrates of bloom dominants enhance the growth of a predecessor, nor do any inhibit the growth of a successor. While this is reassuring, it is not essential to the acceptance of the basic premise of this study. There are many factors involved in the final determination of sequence, and the competitive advantage of metabolite effects could reasonably be overcome by a combination of other factors. It has not, however, been necessary to resort to this rationale, and this absence of incongruous effects for a period of three years suggests that these metabolite effects are not frequently masked.

Table 1. Key

+	Positive; Enhancement
-	Negative; Inhibition
0	Neutral
?	Uncertain; Untested
.....	No Occurrence

TABLE 1. Correlations Between Bloom Sequence and Filtrate Effects

Bloom Sequence 1971-1974 (Dominant Organisms in Order of Appearance) Months of Occurrence	Predecessor and Effect of Dominant on Predecessor as Determined by Bioassay of Filtrates	Successor and Effect of Dominant on Successor as Determined by Bioassay of Filtrates	Contemporaries and Effect of Dominant on Contemporaries as Determined by Bioassay of Filtrates
Oscillatoria rubescens (535) NDJFMA	. . . .	(739) 0 (538) +	. . . .
Oscillatoria rubescens (739) AMJJ	(535) 0	(538) + (762) ? (535) 0	(538) + start
Anabaena sp. (538) Jun.	(739) -	(762) ? (535) +	(739) - end
Anabaena sp. (762) JAS	(739) -	. . . .	(535) - end
Oscillatoria rubescens (535) JAS(O)	(739) 0	(597) + (776) +	(762) ? (597) + start
Pseudanabaena galeata (597) O	(535) 0	(776) 0	(776) 0
Oscillatoria sp. (776) ND(J)	(535) - (597) -	. . . .	(597) - end
Synechococcus sp. (91) AM	. . . .	(765) +	. . . .
Anabaena sp. (765) JJA(S)	. . . .	. . . .	(762) ?
Anabaena sp. (762) A(S)	(765) -	. . . .	(765) - end

The events listed in Table 2 provide examples of another form of correlation, that of year-to-year variations in seasonal patterns which parallel filtrate effects. Negative filtrate effects correspond to the absence of organisms which had dominated the lake during the same season, but in a different year. This exclusion is of the same form as the diatom exclusion discussed below.

The question of the population density of *in situ* blooms and that of *in vitro* filtrate cultures is of great significance to the propriety of the extrapolation from *in vitro* effects to *in situ* occurrences. Population counts were taken for each of the filtrate producing cultures. The maximum *in situ* population and the range of *in vitro* populations are compared in Table 3. Since those effects which influence a competitive situation in nature need only subtly influence a single constituent of that situation and since, in contrast, any *in vitro* effect must be obvious to be accepted as possible, it is assumed that an *in vitro* population density three to four times that of the *in situ* population would not be unreasonably dense for purposes of extrapolation. Actually, the inhibitory effect of blue-green filtrates against diatoms was found to be still present at 33% of its original level (100% F); therefore, an *in situ* population one half to one third of the *in vitro* population could be expected to provide an effect strong enough to be consistently detectable in the F vs A assays. As can be seen in Table 3, most *in vitro* population densities were within reasonable limits. Only the *Pseudanabaena galeata* (597) cultures (8-13x), and one of the *Synechecoccus sp*. (91) cultures (160x) produced comparatively dense *in vitro* populations. This low population density reflects measures taken to avoid excessive growth in laboratory cultures. Lake water with a very minimal nutrient addition (1½% ES_I) was employed as the basic culture medium. The use of artificial media, while essential to some experimental design, was not desirable during this study.

III:II THE CORRELATION OF BOTH BLOOM SEQUENCE AND FILTRATE EFFECTS WITH THE BIOLOGICAL ACTIVITY OF LINSLEY POND WATERS

This correlation, because it represents a tri-fold comparison, is more difficult to document and to graphically communicate than is that between filtrate effects and sequence only. As indicated in Table 4, much of the information essential to a thorough exploration of these multi-faceted correlations is lacking. However, for the pond water samples which were tested many events which clearly show a correspondence between sequence-filtrate data (Table 1, Section III:I) and pond water data were observed. The following examples are of special interest.

September 5, and September 19, 1972

Pond water from September 19 inhibited the growth of *Oscillatoria rubescens* (535) *in vitro*. In 1971 this organism had produced a bloom population before turnover and this bloom population maintained dominance

TABLE 2.

SEASONAL VARIATIONS WHICH CORRELATE WITH FILTRATE EFFECTS

ORGANISM	TIME OF ANTICIPATED BLOOM	CORRELATION BETWEEN FILTRATE EFFECT AND IN SITU EVENT
Oscillatoria rubescens (535)	Onset: August Duration: August—April	1973 Anabaena sp. (765) bloomed just prior the anticipated onset of the (535) bloom, No (535) bloom occurred. Filtrate: no filtrate was produced by (765), but the unialgal bloom in the pond was exceedingly dense and water collected at the peak of the (765) inhibited growth of (535)
Oscillatoria rubescens (739)	Onset: April Duration: April-July	1973 Synechecoccus sp. (91) bloomed at the time of (739) onset. No (739) bloom occurred. Filtrates of (91) inhibit growth of (739).
Anabaena sp. (538)	Onset: early June Duration: June	1973 Synechecoccus sp. (91) bloomed just prior to the anticipated onset of the (538) bloom. No (538) bloom occurred. Filtrates of (91) inhibit growth of (538). 1973 Anabaena sp. (765) bloomed at the anticipated time of the (538) bloom. No (538) bloom occurred. (765) water inhibited (538), see (535) above for explanation.

TABLE 2 (continued)

SEASONAL VARIATIONS WHICH CORRELATE WITH FILTRATE EFFECTS

ORGANISM	TIME OF ANTICIPATED BLOOM	CORRELATION BETWEEN FILTRATE EFFECT AND IN SITU EVENT
Synechococcus sp. (91)	Onset: April Duration: April, May	1972 Oscillatoria rubescens (739) bloomed at the anticipated time of the (91) bloom. No (91) bloom occurred. Filtrates of (739) inhibit growth of (91) in culture*.
Anabaena sp. (765)	Onset: June Duration: June-August	1971 Anabaena sp. (538) bloomed at the anticipated time of the (765) bloom. No (765) bloom occurred. Filtrates of (538) inhibit growth of (765) in culture. 1971 Oscillatoria rubescens (739) bloomed at the anticipated time of the (765) bloom. No (765) bloom occurred. Filtrates of (739) inhibit growth of (765) in culture.

* (91) and (739) may be mutually exclusive. Filtrates do not suggest that the preceding organisms determine which will bloom. In 1971 the Predecessor was O. rubescens (535) which is neutral to both. In 1972 there was no preceding bloom.

TABLE 3.

COMPARISON OF IN SITU AND IN VITRO POPULATION DENSITIES
(Listed in thousands of organisms per ml)

ORGANISM	IN SITU POPULATION MAXIMUM*	IN VITRO POPULATION RANGE*
Oscillatoria rubescens (535)	16	4-55
Oscillatoria rubescens (739)	17	12-21
Anabaena sp. (538)	6	11-33
Pseudanabaena galeata (597)	5	40-63
Oscillatoria sp. (776)	9	7-17
Synechecoccus sp. (91)	1000	460-160,000
Aphanizomenon flos-aquae (766)		11-12
Anabaena sp. (762)	13	12

* Filamentous forms calculated at a length of 0.4 mm.

TABLE 4.

TRIFOLD CORRELATIONS: BLOOM SEQUENCE; FILTRATE EFFECTS; AND BIOLOGICAL ACTIVITY OF COLLECTED LINSLEY POND WATER SAMPLES

SYMBOLS

. . = no occurrence

? = effect unknown

+ = positive effect-enhancement

- = negative effect-inhibition

0 = no effect noted

( ) = parentheses enclosing effect sign means effect determination unclear

++ = very strong positive effect-enhancement

TABLE 4. TRIFOLD CORRELATIONS: BLOOM SEQUENCE; FILTRATE EFFECTS, AND BIOLOGICAL ACTIVITY OF COLLECTED LINSLEY POND WATER SAMPLES

DATE OF POND H_2O	DOMINANT ORGANISM	SUCCESSOR	EFFECTS ON SUCC. of		PREDECESSOR	EFFECTS ON PRED. of	
			POND H_2O	FILT. OF DOM.		POND H_2O	FILT. OF DOM.
8/1-8/8/72 Fresh	*Oscillatoria rubescens*(535) max.	. .	. .	. .	*Oscillatoria rubescens* (739)	-	(0)
					Anabaena sp. (762)	?	?
	Anabaena sp. (762) max.	. .	. .	. .	*O. rubescens* (739)	?	-
9/5/72 Fresh	*Anabaena* sp. (762) end	*Pseudanabaena galeata* (597)	?	- interval	*Oscillatoria rubescens* (535)	-	-
	Oscillatoria rubescens(535)	*Pseudanabaena galeata* (597)	?	+	*Anabaena* sp. (762)	?	?
					Oscillatoria rubescens(535)	?	(0)
9/19/72 Thaw	*Anabaena* sp. (762) end	*Pseudanabaena galeata* (597)	0	- interval	*Oscillatoria rubescens* (535)	?	-
	Oscillatoria rubescens(535)	*Pseudanabaena galeata* (597)	0	+	*Anabaena* sp. (762)	?	?
					Oscillatoria rubescens(535)	-	(0)
11/17/72 Thaw	*Oscillatoria* sp. (776) max.	*Oscillatoria* sp. (776)	-	(0)	*Pseudanabaena galeata* (597)	-	-

TABLE 4. (continued)

DATE OF POND H_2O	DOMINANT ORGANISM	SUCCESSOR	EFFECTS ON SUCC. OF		PREDECESSOR	EFFECTS ON PRED. OF	
			POND H_2O	FILT. OF DOM.		POND H_2O	FILT. OF DOM.
1/7/73 Fresh	Oscillatoria sp. (776) late	Flagellates	(+)	mixed	Oscillatoria sp. (776)	+	(0)
3/18/73 Fresh Thaw	Flagellates	Flagellates	mixed	. .	Flagellates	mixed	. .
	Asterionella formosa (800)	Asterionella formosa (800)	+	. .	Oscillatoria sp. (776) late	0	. .
	Oscillatoria sp. (776) end	Oscillatoria sp. (776) late	(0)	. .			
		Synechecoccus sp. (91)	+	. .			
5/13/73 Fresh	Synechecoccus sp. (91) max.	Asterionella formosa (800)	-	-	Asterionella formosa (800)	-	-
		Anabaena sp. (765)	?	++			
6/2/73 Fresh	Asterionella formosa (800) end (second)	Anabaena sp. (765)	++	?	Synechecoccus sp. (91)	0	?
7/17/73 Thaw	Anabaena sp. (765) max.	Anabaena sp. (762) weak bloom	?	?	Anabaena sp. (765)	++	?
8/13/73 Fresh	Anabaena sp. (765) end	Clear water for over 3 mos.	. .	. .	Anabaena sp. (765)	0	?

TABLE 4 (continued)

DATE OF POND H_2O	DOMINANT ORGANISM	SUCCESSOR	EFFECTS ON SUCC. OF		PREDECESSOR	EFFECTS ON PRED. OF	
			POND H_2O	FILT. OF DOM.		POND H_2O	FILT. OF DOM.
2/14/74	Oscillatoria sp. (776) low	Diatoms	-	. .	Oscillatoria sp. (776) very low	-	
	Pseudanabaena galeata (597) low	Flagellates	mixed	. .	Pseudanabaena galeata (597) very low	-	
	Flagellates high						
	Diatoms, high						
6/13/74	Oscillatoria rubescens (535)	Oscillatoria rubescens (535)	+	0	Diatoms	-	-
					Flagellates)		
					(monad) (317)	+	+
					Chlamydomonas (298)	-	-
					Sphaerellopsis (598)	-	?
					Synura uvella (43)	-	0

in the pond throughout the 1971-1972 winter. When O. rubescens (535) appeared in late summer 1972, it was expected that it would again produce a bloom population which would dominate through the winter (1972-1973). The basis for the sudden demise of this bloom early in September was explored. No sure explanation was found. Pond waters, however, were consistently inhibitory to (535). Pond waters from September 5 and September 19 were found to be inhibitory to Anabaena sp. (762), Aphanizomenon flos-aquae (766), Anabaena sp. (765), and Anabaena circinalis (769), all of these were blue-greens which had been present in the water at the end of August, 1972. Thus the pond water exhibited in vitro the same generalized inhibition of blue-greens which sequential events in the pond had displayed in situ. It is probable that inorganic nutrient limitation played a part in the rapid drop in phytoplankton in early September since both F and A assays produced less than expected population densities (as compared to experimental cultures in waters collected in other seasons). However, nutrient limitation alone cannot account for F vs A differences since F and A inorganic nutrient conditions are the same.

November 17, 1972

Waters from this date inhibited the growth of Pseudanabaena galeata (597) in vitro. The dominant organism at this time was Oscillatoria sp. (776). Oscillatoria sp. (776) filtrates also produced inhibition of the in vitro growth of P. galeata (597). Since the (776) bloom replaced a (597) bloom, the pond water and filtrates apparently exhibited in vitro the same inhibition of this organism as did the sequence in situ.

March 18, 1973

In contrast to its usual universally negative effect on diatom growth, this sample of Linsley water enhanced the in vitro growth of Asterionella formosa (800) only, and this sample was taken at the time of the first diatom bloom of the study period—a bloom dominated by A. formosa (800).

May 13, June 2, July 17, and August 13, 1973

In 1972 Oscillatoria rubescens (739) bloomed in early spring and Anabaena sp. (538) bloomed in late spring; but in 1973 Synechecoccus sp. (91) bloomed in early spring and Anabaena sp. (765), which is quite different morphologically from (538), bloomed in late spring.

Filtrates of Oscillatoria rubescens (739) enhanced growth of Anabaena sp. (538) and inhibited growth of Anabaena sp. (765). Filtrates of Synechecoccus sp. (91) enhanced growth of Anabaena sp. (765) and inhibited growth of Anabaena sp. (538). Thus it appears that both enhancement and exclusion are involved in the determiantion of bloom sequence in the springs of 1972 and 1973.

Further, filtrates of Anabaena sp. (538) inhibited the growth of Anabaena sp. (765). Unfortunately, the only bloom dominant which never produced a filtrate for bioassay was Anabaena sp. (765), but the pond waters from July 17, 1973, during an immense, essentially unialgal, bloom of Anabaena sp. (765) inhibited the growth of Anabaena sp. (538). Thus a mutual exclusion also appears to be involved in the determination of bloom sequence in the springs of 1972 and 1973.

Pond waters from June 2, 1973 taken after the bloom of Synechecoccus sp. (91), enhanced the in vitro growth of Anabaena (765). Pond water from July 17, six weeks later, was still able to enhance in vitro growth of Anabaena sp. (765). At that time the in situ bloom of Anabaena (765) was at its maximum. It remained at maximum for approximately one more week.

By August 13, the (765) bloom had subsided, and the pond waters were quite free of any form of blue-green bloom. Pond waters from that date were found to be neutral to the growth of (765) in vitro; however, a lag in the growth of both F and A tests was noted. Both F and A cultures were less dense than those in pond water collected a month earlier, suggesting depletion of nutrients in late summer 1973 similar to that in late summer 1972.

Anabaena sp. (765), while it grows very slowly in maintenance culture, readily produces dense cultures in the filtrates of Synechecoccus sp. (91) and in pond waters taken during or immediately after blooms of Synechecoccus sp. (91). The addition of a small amount of the cell-free filtrate of Synechecoccus sp. (91) was found to provide a more satisfactory maintenance culture for (765). The heat-stable factor responsible for this phenomenon was not isolated.

In conclusion, although the correlation of filtrate effects, sequential events, and pond water effects cannot assure that the same stimuli are producing similar responses, the multiplicity of these correlations strongly supports such an assumption. It should be noted that in most respects frozen water samples gave results similar to those from freshly collected samples, but the possibility that some changes may occur during the freeze-store-thaw process cannot be ignored (Table 8; Section VII:II). Therefore, it is suggested that future studies of correspondence between in vitro and in situ events should be done using freshly collected samples of pond water. This would provide more reliable bioassays.

SECTION IV

WATER QUALITY MANAGEMENT PROSPECTS:

PROGRAMMING BLOOM SEQUENCE

The information developed during this study suggests a new approach to water quality management in moderate-sized eutrophic lakes—sequence control. This relatively inexpensive method, if successful, would offer a practical alternative to sewage treatment and phosphate elimination.

The information in Section IV:I is the basis for the hypothetical sequence control plan for Linsley Pond outlined in Section IV:II. This information includes data specific to Linsley and data of a general nature for fresh waters. The plan represents an attempt to replace some of the blue-green blooms in Linsley by diatom blooms because the diatoms are aesthetically (taste, smell, touch) and ecologically (strong link in food chain) preferable to blue-greens. They also reduce the available nutrients in lake water by carrying nutrients down into the hypolimnion as they senesce and these nutrients are not recycled until after the fall overturn. In contrast blue-greens lyse in the epilimnion, releasing their nutrients for immediate recycling by subsequent blooms.

Improving conditions for the zooplankton community by increasing their food supply and by eliminating some of the blue-greens should, in turn, produce a healthy fish population. This chain of events occurred naturally in Linsley in 1973, and is discussed in Section VI:IV.

IV:I *IN SITU* EXCLUSION BY BLUE-GREEN METABOLITES

Probable Exclusion of Diatoms by Blue-Greens

1) Spring diatoms which commonly produce blooms in oligotrophic and mesotrophic lakes require high N, P, and Si and they develop their blooms at low temperatures during short days.

2) Diatom growth in Linsley has apparently been prevented in the spring by the accumulation of metabolites produced by blue-greens growing during the winter. When blue-green growth was low in winter, spring diatom blooms developed (Figure 12, Section VI:V).

3) Preliminary evidence suggests that the addition of $Na_2SiO_3 \cdot 9H_2O$ can overcome this inhibition.

4) Spring diatom blooms which occurred in Linsley ended when available silica was low. Available silica need not equal measured, dissolved inorganic silica; see VII:V.

Probable Exclusion of Zooplankton by Blue-Greens

1) Zooplankton populations do not develop in Linsley when the waters are dominated by blue-green blooms.

2) Zooplankton populations which develop when blue-greens do not dominate Linsley waters rapidly disappear with the onset of blue-green bloom conditions.

3) Preliminary tests indicate that the cell-free filtrates of blue-green algae may inhibit the growth of zooplankton. Also, other investigators have shown that blue-greens are in general a poor food choice for zooplankton.

IV:II A STRATEGY FOR SEQUENCE CONTROL

1) Winter blue-green blooms should be eliminated by a commonly accepted algicide. $CuSO_4$ might be applied immediately after overturn. This would remove the cause of spring diatom bloom exclusion. While a second treatment might be considered if a late winter blue-green bloom develops, it may be sufficient to eliminate some, rather than all, blue-green winter growth.

2) Spring diatom growth should be enhanced by the addition of silica. Silica would lessen the effect of remaining blue-green metabolite inhibition and would extend the diatom bloom when natural silica was depleted. Because it is a fertilizer only for diatoms and a few other desirable forms, it would not stimulate unwanted growth.

3) These efforts should increase the period of diatom domination and decrease nutrient levels left in the epilimnion when the diatoms senesce. Ideally, this would shorten any subsequent (late summer) blue-green bloom and would decrease its density.

4) Zooplankton growth should be significantly improved by the decrease in antagonistic organisms (blue-greens) and the increase in food organisms (diatoms and other desirable forms).

SECTION V

INTRODUCTION

V:I EUTROPHICATION

Widespread, excessive eutrophication (Hutchinson, 1969) of fresh waters, which is a direct result of either inadequate waste water treatment or of contamination by runoff waters (Biggar and Corey, 1969; Cooper, 1969; Weibel, 1969), is presently controlled either by measures aimed at the elimination of bloom-stimulating waste products by tertiary waste water treatment and a concomitant modification in land use practices, or by measures intended to eliminate the bloom organisms themselves via the application of chemical herbicides (Mulligan, 1969). Successful control with the former approach is not only exceedingly expensive (U. S. Department of the Interior, 1968), but is also dependent on the cooperation and compliance of great numbers of individuals, corporations, and local, national and international governing bodies (U. S. Government Printing Office, 1972); and success with the latter, chemical, approach is fraught with unknown ecological repercussions (Crosby and Tucker, 1966).

Phosphate Control—Selected Successes

The success of Edmondson (1972) in the Lake Washington project settled the question of the practical value of eliminating phosphate from inflow waters, even if no parallel attempt to eliminate the standing load of phosphate in lake waters and, or, sediments is made. This success also indicates that a real prospect of rapidly reversing the conditions of cultural eutrophication exists (Hasler, 1947; Tanner, et al., 1972) if wholesale phosphate removal (from inflow), diversion, precipitation, etc., is undertaken.

In a series of whole lake experiments in Ontario Schindler (1974) controlled the level of algal bloom by various regimes of nutrient addition and concluded that in such natural lake waters phosphate addition alone is capable of initiating algal bloom conditions. By dividing a single lake in two with a plastic and nylon sea curtain he demonstrated that while additions of nitrate, phosphate, and organic carbon do produce extraordinary bloom development, additions of only nitrate and organic carbon produce no changes in the usual flora of the lake; i.e., a population of mixed diatoms. Unfortunately, these accomplishments do not alter the fact that in many cases mechanical or chemical phosphate elimination is unrealistic, representing untenable public expense.

Legislation banning high phosphate detergents in New York State (Murphy, 1973) has led to marked improvement in the degree of cultural eutrophication observed in Onondaga Lake and suggests a reasonably inexpensive method of control. In spite of such attendant drawbacks as: 1) the requirement of long term cooperation of the public in not purchasing "bootleg" high phosphate detergents due to the lack of a safe and effective substitute; and, 2) the likelihood that non-detergent phosphate sources alone may in some waters contribute sufficient phosphate to support severe cultural eutrophication—such legislative action may provide substantial improvement in many small, urban-locked, bodies of water.

Biological Control

In contrast, biological control is highly desirable both as a more ecologically sound alternative to the use of chemical herbicides and as a practical adjunct to waste water treatment and phosphate control. Biological control, however, requires an intimate knowledge of the many physical and biological factors which promote and sustain the natural sequence of bloom populations, and this information is presently insufficient in both quantity and scope.

Established Parameters of Bloom Sequence Control

Bloom sequence has been shown to depend in part on such factors as:

-total solids (Provasoli, *et al*., 1954; Provasoli and Pintner, 1960);
-photoperiod (Dunshaw, 1973; Reynolds, 1973);
-light intensity (Ignatiades and Smayda, 1970a; Sorokin and Krauss, 1958);
-accumulation of pesticides (Cope, 1962; Mensel, *et al*., 1970; Wurster, 1968);
-salinity (Ignatiades and Smayda, 1970a);
-temperature (Conover, 1956);
-carbondioxide-pH regime (Brock, 1973; Goldman, 1973; Shapiro, 1973);
-availability of nutrients (Chu, 1942, 1943); especially of silica (Hamilton, 1969) and trace metals (Horne and Goldman, 1974; Ignatiades and Smayda, 1970b).

Certain biological factors are also considered significant in the determination of qualitative and, or, quantitative characteristics of bloom populations. Among these are:

-buoyancy regulation (Fogg and Walsby, 1971);
-organic micronutrients (Carlucci, 1974; Carlucci and Cuhel, 1974; Carlucci and Shimp, 1974; Hagedorn, 1971a, 1971b; Owada, *et al*., 1972; Provasoli, 1960; Schwartz, 1972);
-selective zooplankton grazing (Porter, 1973);
-pro- and antibiotic effects of extracellular metabolites of, and the direct competition for nutrients of, vascular plants (Fitzgerald, 1969; Hasler and Jones, 1949; Wetzel, 1969; Wetzel and Allen, 1971).

In the last decade (Safferman, 1964; Shilo, 1971, 1973; Stewart, 1969) the possibility that viruses may be common in blue-green algae has prompted studies into the possibility of selectively employing cyanophages to eliminate blooms. During the discussions following both his 1971 and 1973 papers, Shilo expressed doubt as to the feasibility of the controlled use of cyanophage infection in this manner. His conclusions were based on the specificity of the phages as compared to the variety of blue-greens in most natural waters and to capacity of these algae to produce additional phage resistant mutants. Still, M. Kraus (personal communication, 1973) is of the opinion that cyanophage occurrence is so ubiquitous that they must be considered a possible factor in the determination of the natural complement and density of blue-green blooms.

To date, these many physical, chemical, and biological parameters, although they have been shown to be significant in the specific cases referenced above, have not provided sufficient explanation for bloom sequence in general (Fogg, 1953, 1966, 1971; Hutchinson, 1941, 1944, 1971 lecture; Lucas, 1961); therefore, additional control mechanisms must be sought. The effects of the metabolic products of bloom dominant organisms on bloom sequence is explored in this study.

V:II AN HYPOTHESIS

Perspective

The involvement of biological effects in the determination of species sequence was first suggested by Apstein in 1896 and reiterated by Putter in 1908. Yet for decades the possibility that biological factors may modulate bloom sequence in freshwater communities was neglected until Lucas (1947) postulated that external metabolites might be ecologically important.

That algae produce extracellular metabolites (Fogg and Boalch, 1958; Forsberg and Taube, 1967; Schwimmer and Schwimmer, 1964; Steeman-Nielsen, 1952; reviews by Fogg, 1962, 1966; Lucas, 1947; Saunders, 1957), and that these metabolites can effect the growth of other algae (Carlucci and Bowes, 1970a, 1970b, 1972; Droop, 1968; Lefevre, *et al*., 1952; Pratt, 1966; Proctor, 1957; reviews by Hartmann, 1960; Pourriot, 1966) are now established facts. However, among the many studies of interspecific metabolite effects there are very few attempts to demonstrate a correlation of *in vitro* activity with *in situ* occurrence. Rather, since the bulk of this work deals directly with *in vitro* phenomena, the implied probable *in situ* significance of these effects encounters the many pitfalls inherent in such generalizations (Hutchinson, 1966).

Basic Premise and Model

The basic premise of this study is that some, or all, of the organisms which dominate freshwater blooms produce biologically active extracellular metabolites in sufficient quantity and of appropriate quality to effect the sequence of blooms in situ. Figure 1 represents a simple model based on this premise. That is, if organisms A, B, and C produce blooms in the order a, b, c, then several distinct, but synergistic, metabolite effects may be expected.

Inclusive:

I. It is likely that organism A produces an extracellular metabolite which will *enhance* the growth of organism B in such a manner as to improve the competitive position of B, thus encouraging a bloom of organism B (among the many possible candidates) to replace the original bloom. Subsequently, it is also likely that a similar chain of events would encourage organism C to replace organism B.

II. It is likely that organism B produces an extracellular metabolite which will *inhibit* the growth of organism A in such a manner as to improve the competitive position of B, thus encouraging a bloom of organism B (among the many possible candidates) to replace the original bloom. Subsequently, it is also likely that a similar chain of events would encourage organism C to replace organism B.

Exclusive:

III. If organism A produces an extracellular metabolite which inhibits a second organism, X, which might otherwise be expected to bloom at the time (season) immediately following organism A, it is likely that the competitive position of organism X would be sufficiently damaged so as to eliminate, or exclude, a bloom of organism X from the in situ sequence. Diatom exclusion is an example of this phenomenon; Sections VII:II and, especially, VII:III and VII:IV.

It is not suggested that this combination of enhancement and inhibition is solely responsible for bloom sequence, but rather that it is one of the significant contributors to the determination of bloom sequence in general.

Experimental Design

To give greater credence to the extrapolation of in vitro results to the explanation of in situ phenomena three different approaches to the problem of metabolite involvement in bloom sequence determination were employed.

I. The in situ physical and chemical conditions, and the natural bloom sequence of the study pond (Linsley), were monitored for a

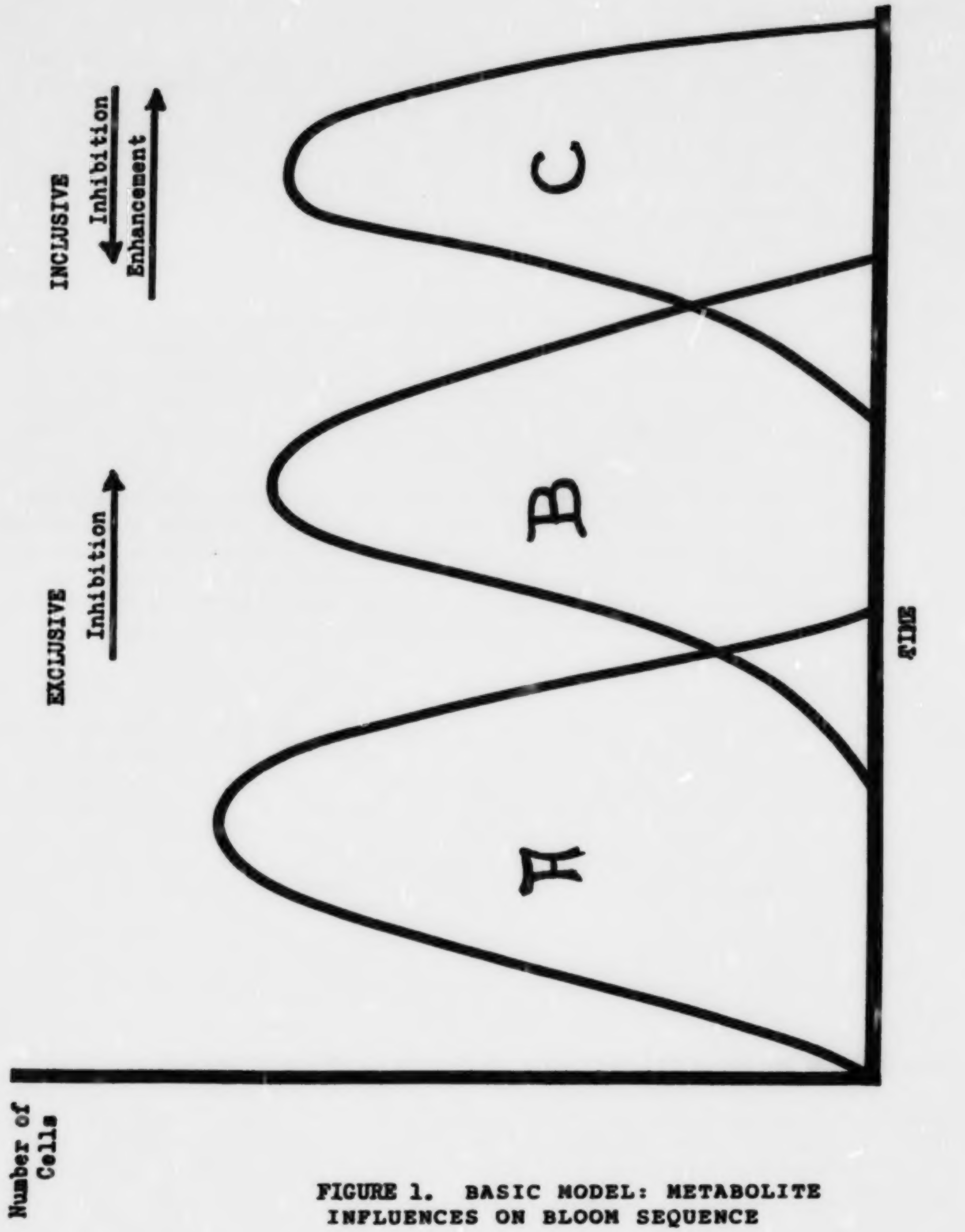

Number of Cells
EXCLUSIVE
Inhibition
INCLUSIVE
Inhibition
Enhancement
A
B
C
TIME

FIGURE 1. BASIC MODEL: METABOLITE INFLUENCES ON BLOOM SEQUENCE

period of three years to determine if changes in the usual limnological parameters were accompanied by specific changes in bloom sequence, Section VI.

II. Those algae which were observed to be dominant in blooms during this three year monitoring period were isolated, established in culture (axenic when possible), and employed to produce relatively large batch cultures. Batch cultures were harvested by passage through a 0.45µ Millipore filter, and the resulting cell-free filtrates were bioassayed for biological activity which might parallel *in situ* events, Sections III and VII.

III. Large water samples were taken during periods of peak bloom and these samples were bioassayed in the same manner as were the cell-free filtrates for biological activity which might parallel *in situ* events, Sections III and VII.

Special Problems Associated with Algal Metabolite Studies

Certain problems with the interpretation of algal metabolite studies are especially frequently encountered. For example: the practice of employing algal species from culture collections. Because these organisms are generally isolated from different environments, the extrapolation of interspecies effects from *in vitro* experiments to specific *in situ* occurrences is tenuous at best.

During this study most of the algae, filtrate producers and assay organisms, were isolated directly from Linsley Pond. The only exceptions were organisms intentionally chosen as "non-Linsley" algae for tests designed to provide information concerning the possible extrapolation of the conclusions of this study to fresh waters other than Linsley. It is of some interest that the very consistent pattern of diatom inhibition by heat-labile metabolites of blue-greens was not as obvious when a set of experimental organisms from several different sources was used.

Another problem, which develops when organisms are fresh isolates, is that they are often established in bacterized culture—with the effect that no distinction can be made between the effects of algal and bacterial metabolites. It is true that in some instances employing bacterized algal cultures is unavoidable because it is not always possible to separate an alga (especially a blue-green) from its accompanying bacterial community. However, since the bacterial population, and thus its influence on the system, may radically change when the natural community is transferred from a lake to a test tube, efforts must be made to distinguish algal from bacterial effects.

During this study a variety of bacterized and axenic filtrate producers, and bacterized and axenic assay organisms are employed. To aid in the differentiation of algal from bacterial effects a mixed culture of Linsley bacteria was added to an axenic producer culture and results for the subsequent bioassay were compared to results from bioassays of the axenic culture's filtrate.

An additional common criticism of studies relating algal metabolite effects to natural occurrences involves dilution. There is concern that the effects observed in laboratory tests can be accurately interpreted only for *in vitro* circumstances and cannot be extrapolated to the natural situation. This is because the very dense growth in test tube cultures could be expected to produce a far greater concentration of metabolites than could be produced *in situ*, where the relatively large volume of water could dilute the population of producers, and where environmental conditions, especially nutrient levels, are quite different. In turn this would dilute the *in situ* concentration of metabolites to a point where the biological activity demonstrated in the "artificially" concentrated *in vitro* tests could not be significant. Artificial media often provide quite unnatural levels for certain nutrients, especially for the trace elements whose absolute quantities may be critical to *in situ* growth but very difficult to measure or to reproduce.

During this study population densities in producer cultures were similar to those of the natural waters (Table 3, Section III:I). Since enriched natural pond water was used as the basis for experimental media, *in vitro* levels for most trace elements are assumed to be similar to *in situ* levels.

V:III SIGNIFICANT RELATED RESEARCH

Algal Metabolites In Situ

In two extensive studies relating either to salt marsh (Lee, *et al*., 1970) or ocean (Aubert, 1963-1971) communities, the involvement of algal extracellular metabolites in a variety of ecologically significant phenomena was investigated. Bloom sequence determination was not among the major interests of either study; however, the conclusions in both that metabolites are present in salt marsh and, or, ocean waters in sufficient quantity to produce observable biological repercussions (especially Aubert's bactericidal effects) weigh strongly against the dilution argument.

A significant proof of the importance of extracellular metabolite effects in the determination of dominance in freshwater algal blooms was provided by Williams (1971) when he presented conclusive evidence that an extracellular inhibitor is produced by *Anabaena flos-aquae*, and that this substance is in part responsible for the periodic dominance of Lake Nelson, New Jersey, by *A. flos-aquae*. Because his primary goal was to demonstrate metabolite involvement in a single bloom situation in nature, field study covered only that short period during which *A. flos-aquae* dominated the lake waters. He offered a strong basis for his conclusions with parallel results obtained from *in situ* and *in vitro* studies.

Antibacterial Effects of Algal Extracellular Metabolites

Aubert, et al. (1963-1971), have developed a strong in vitro case for the algal antibacterial effect they believe is demonstrated in situ by rapid dissipation of sewage bacteria in the Mediterranean Sea. Though they have included the algae among those organisms secreting antibacterial substances, they have not excluded the possibility that their role may be minor in comparison to the role of marine bacteria, as Moebus (1972a, 1972b, 1972c) has suggested, or to the more commonly proffered explanations of anti-coliform activity which consider sedimentation, predation, and lack of reproduction to be the critical factors.

Sieburth (1964) with less in vitro evidence has tied the anti-coliform activity of sea water samples from Narragansett Bay to the irregular blooms of *Skeletonema costatum* and has presented a less strenuous in vitro argument to substantiate his thesis; i.e., his cultures require bacteria and additional phytoplankters to develop a strong antibiotic effect (his axenic cultures of *S. costatum* show no anti-coliform activity).

The work of several others has also served to substantiate the in situ antibacterial activity of algae. Steeman-Nielsen (1955), using light and dark bottle techniques to measure oxygen consumption, noted that the algae growing in "light" bottles inhibited bacterial respiration and thus interfered with measurement techniques. Jørgensen (1962) isolated chlorophyllides from filtrates of a variety of naturally growing algae and found them to be inhibitors of bacterial growth. Jones (1959) found the soluble organic extracts of sea water collected during a "red tide" bloom of *Gonyaulax polyhedra* to be highly antibacterial. Interestingly, he noticed a zone of stimulation surrounding the zone of inhibition on agar plates which is reminiscent of certain effects of plant hormones (Bentley-Mowat and Reid, 1969), indicating stimulation at low concentrations, and inhibition at high concentrations. This is only one example of the complicated array of activity to be expected from algal metabolites. This might also have been the dilution-stimulation Pratt (1942) thought he was witnessing in his *Chlorella* experiments; or it might have been several extracellular metabolites as Jørgensen and Steeman-Nielsen (1961) and Jørgensen (1962) proved Pratt's "Chlorellin" to be; or it might have been an indication of some totally unknown phenomenon peculiar to this alga.

In 1966 Duff, Bruce and Antia surveyed the antibacterial range and potency of Bacillariophyceae, Chrysophyceae, and Cryptophyceae and concluded that these are more generally potent antibacterially than are the Chlorophyceae or the blue-greens. Their algal samples, used after drying as a source of active substance, were harvested from axenic mass cultures derived from a variety of geographic origins. The assay bacteria were from type collections, or were fresh isolates from the sea. Two ecologically significant theses were postulated: 1) the selective activity of their algal stains against Gram-positive bacteria (especially

Staphylococcus) may account for the prevalence of Gram-negative bacteria in the seas (also suggested by the work of Saz, et al., 1963); and 2) the specific value of the antibacterials, in terms of natural selection, may be to control epiphytic bacteria. This epiphytic association would eliminate the aforementioned dilution arguments against in situ antibiotic effects.

Other work with epiphytic associations includes that of Fitzgerald (1969) who found that Cladophora in nitrogen-poor situations were free of epiphytes (including other algae). An evolutionarily selective value may be found in the coincident excretion of substances toxic to epiphytes at that time when the Cladophora is experiencing nutritional stress. Similarly, McLachlan and Craigie (1964) found that Fucus vesiculosis produced an inhibition of unicellular algae (likely epiphytes and competitors), and Jørgensen (1956) demonstrated an inhibition by planktonic diatoms and Chlorophyta on epiphytes.

In 1970 Davis and Gloyna determined that axenic cultures of freshwater green and blue-green algae were mildly inhibitory to enteric bacteria and decidely inhibitory to pathogenic forms. Their parallel experiments in waste stabilization ponds produced similar results; however, the endemic bacterial communities of these ponds provided cultures of Flaveobacterium and Brevibacterium which, they noted, were more effective than the algae tested in eliminating enteric forms.

Fogg (1962) expressed the opinion that "when vigorously growing cultures of algae are exposed to contamination, it is often observed that relatively few bacteria develop, an effect which could conceivably be due to their suppression by antibacterial agents". He was, nevertheless, still dissatisfied with the experimental proof of the importance of in situ algal extracellular metabolites in 1966; and in 1971 he indicated (Fogg and Walsby) additional dissatisfaction with all prior explanations of blue-green dominance of blooms in eutrophied waters and offered, instead, a partial explanation on buoyancy.

Concentration, Dilution, and Identification of Algal Metabolites

When the effects of toxic "red tides" (Gilbert, 1974) and the effects of the "fast death" factors produced by certain strains of Microcystis aeruginosa and Anabaena flos-aquae are considered (Gorham, 1964; Fogg, 1962), it is reasonable to assume that algal extracellular metabolites do occur in sufficient quantities in situ to cause metabolic reactions in other aquatic organisms. Schwimmer and Schwimmer, in 1964, offered an intriguing discussion of the various documentations of algal toxicosis in vertebrates, including humans. More recently Quick (1973) found the "whirling death" fish kills in Biscayne Bay to be due to the presence of Anacystis sp., and Aziz (1974) obtained a diarrhea toxin from a new strain of M. aeruginosa. However, proofs relative to in situ potency are few and, as such, they provide a basis neither for generalization, nor for the assumption that biologically active algal metabolites do commonly produce effects on other algae.

This present study considers the possibility that algal metabolites may be sufficient in quantity, and appropriate in quality, so to influence the growth and reproductive rates of other algae that the presence of a bloom population of a "producer" species or strain (for strains differ biochemically, Gorham, 1964) will determine the presence or absence of a bloom population of some other, "sensitive", species.

A warning concerning the peculiarities of nature and our inability to reconstruct its complicated array of variables is inherent in Fogg's (1966) caution (a) that stationary numbers in nature and in culture may not be the overt manifestation of similar phenomena; and (b) that the massive release of some metabolites "occurs only under particular circumstances which do not necessarily occur in laboratory cultures, but which may occur regularly under natural conditions". This latter warning follows his commentary on earlier experiments with glycolic acid excretion, wherein natural marine and fresh waters showed the results of very high excretions of fixed carbon while cultures showed insignificant levels (below).

Duff, et al., (1966) concluded that the green algae are generally less active antibiotically than other forms, thus substantiating Krogh's (1930) early work indicating a frugality in green algae which he generalized to the other algal forms. This lack of evidence for the excretion of large quantities of metabolite does not suggest a lack of activity. One need only consider the microquantities of plant hormones necessary to promote growth, flowering, abscission, etc., to appreciate this.

Berland, et al., (1972) found that in order to demonstrate an antibacterial effect on their most sensitive assay organism in filtrates of Stichochrysis immobilis, they had to use a twenty-fold concentration of the filtrate. Kroes (1971, 1972) found that a ten to twenty-five-fold concentration was necessary. He concluded that the four types of extracellular inhibitors he found were not ecologically significant because of this concentration requirement and suggested pH effects as the really significant factor in supposed inhibitory effects. To properly consider the doubts raised by concentration requirements one must consider Fogg's warnings (1966, above) and the more recent report of Ignatiades and Fogg (1973) that, depending on culture conditions, anywhere from 2.1% to 87.4% of the total carbon fixed by a single organism may be excreted.

In order to present parallel, or proportional, arrays of data from in situ algae and surrounding natural waters, Whittaker and Vallentyne (1957) analyzed lake waters to determine the array of sugars they contained. This grouping (maltose, sucrose, glucose, fructose, galactose, arabinose, ribose, xylose, and two unknowns) of sugars they then compared with sugars found in, or produced by, various modules of the lake ecosystem (mud, free swimming bacteria, larvae, phytoplankton). They concluded that the open water phytoplankton were the only plausible source of these sugars. The phytoplankton produced large quantities of these sugars directly and also produced quantities of polysaccharides which could be easily decomposed into these sugars.

Bentley (1960) found two plant auxins, which behave identically in chromatographic separation, in phytoplankton and in sea water samples. Since this activity pattern significantly differed from that of the products of other organisms from the same waters, she concluded that the auxins in the water sample originated in the phytoplankton. Further proof was provided by Steeman-Nielsen (1952). Using ^{14}C tracers he followed carbon through fixation and out into organic matter filtered from lake water. Many similar experiments by other investigators followed.

A few values, if noted with proper perspective, can be enlightening. Attempts were made as early as 1930 to determine precisely what portion of fixed carbon (or nitrogen) is excreted by various algae. Braarud and Føyn (1930) estimated that a marine Chlamydomonas released approximately 30% of its organic production. This figure compares reasonably with Lewin's 1956 estimate that Chlamydomonas excreted from 40-60% of its total organic product into its mucilaginous capsule, and with the 35-40% excretion estimate of Antia, *et al*., (1963). In contrast Fogg reported (1966) a 95% excretion level in fresh waters under certain circumstances, but found that the more usual levels ranged between 7% and 50% (usually varying inversely with population density). He suggested that similar levels could be expected in marine waters. Hellebust's (1965) *in situ* and *in vitro* estimates were somewhat lower for "healthy" organisms than were those of Fogg, or of Antia. Hellebust distinguished healthy (4-16%) and senescent (17-38%) colonies. In contrast Nalewajko (1966) reported *in vitro* excretion for healthy organisms at less than 2%. However, Nalewajko, used young log cultures and allotted one hour for labelling (early products); while Hellebust, used young log cultures and allotted four days for labelling (full array of products with possible recycling). Thus superficial comparison which suggests great disparity in data can be quite misleading. As Fogg (1962) commented "measurements of the amounts of extracellular material can have no precise significance unless the physiological history of the system and its environmental conditions are defined".

Vitamins as Algal Extracellular Metabolites

Provasoli (1963) suggested three possible areas of *in situ* metabolite activity; 1) the removal, or deactivation, of inhibitions; 2) the production of specific inhibition; and 3) the production of necessary nutrients, or growth factors. Much study has been devoted to a portion of his third category. Vitamins and other "growth factors" are required by more than 50% of the algae (Provasoli, 1963; personal communication, 1975; Saunders, 1957). They are also well established as products of algal metabolism (Robbins, *et al*., 1951; Carlucci and Bowes, 1970a, 1972; Bentley, 1958). Excretion, lysis, and decomposition each play a part in releasing those vitamins produced by large masses of algae in fresh and marine waters. Vitamins are, therefore, more an extracellular metabolite in Lucas's sense than in Fogg's. Fogg limits the term "extracellular metabolite" to those metabolic products which are leaked into the surrounding milieu by healthy, growing cells—an essential limitation in many studies. Lucas's less stringent definition is, perhaps, more suited to the present ecosystem study, wherein the presence and availability of metabolites, more than the mode of production, is significant.

As early as 1943 the cycles of thiamine and biotin in lake waters were studied by Hutchinson (in 1946 he and Setlow added niacin). Sufficient quantities of these vitamins were present in the pond waters studied for the needs of the vitamin requiring algae. In 1956 Cowey found that the B_{12} in the sea is also present at adequate levels and Droop (1957) confirmed abundant B_{12} in a variety of marine habitats; however, his later work with the B_{12} binding factor does suggest that ambient levels may not actually represent available levels (1968).

The biotic origin and utilization of aquatic vitamin stores was confirmed directly *in vitro* by studies of vitamin production by marine bacteria (Burkholder and Burkholder, 1956; Menzel and Spoehr, 1962) and indirectly *in situ* by the many observations that concentrations of vitamins in the open sea are generally lower than they are in coastal waters where the greater portion of primary production is localized. Until 1970, however, the source of these marine and freshwater vitamin stores was believed to be bacterial. It was then that Carlucci and Bowes (1970a, 1970b), by confirming *in vitro* production of vitamins by several species of marine algae, provided a strong argument for the inclusion of algae among not only the users of, but also the contributors to, the *in situ* vitamin pool.

The level of vitamins in natural waters is determined by a balance between producers and consumers, and variations in vitamin levels in the sea have been found to correlate with population increases of vitamin requiring algae. In 1956 Cowey found that a drop in B_{12} concentration coincided with May-June diatom blooms (many diatoms used exogenous B_{12}), and in 1959 Vishniac and Riley observed a drop in B12 levels which parallelled a drop in NO_3 during blooms of *Skeletonema costatum* in Long Island Sound, suggesting a direct correlation between NO_3 utilization in cell growth processes and the consumption of B_{12}.

The widespread occurrence of vitamins in measurable quantities in both marine and freshwater environments (*e.g.*, B12 in Linsley Pond throughout 1973 was approximately 4 mγ%), and the demonstrated capacity of bacteria and algae both to use and to produce these vitamins are clear examples of the extracellular metabolites of one group of organisms being of appropriate quality and sufficient quantity *in situ* to effect the metabolism of a second group of organisms.

SECTION VI

FIELD STUDY

VI:I SITE CHOICE AND SAMPLING

Site Choice

Since Linsley Pond is readily accessible, since it is presently evidencing the effects of cultural eutrophication, and since there is an extensive literature relating to Linsley (see exhaustive bibliography in Cowgill, 1970), it was chosen as the body of natural water about which essential field data would be catalogued and from which experimental organisms and materials would be collected.

Linsley is a medium sized (approximately 94,400 m^2; mean depth 6.7 m; maximum depth 14.8 m—Hutchinson, 1938) kettle lake approximately 25 m above sea level. It drains a basin about 20 times its own area (Riley, 1939). There are approximately 100 single family dwellings in the drainage basin, 11 of which are located close to the water's edge.

The main inflow stream (approximately 2.5×10^4 m^3 wk^{-1}—Hutchinson, 1941), which enters the lake at its southwestern extremity is from Cedar Lake, a slightly larger, though somewhat shallower, body of water which is located in very similar environs less than 500 meters to the east of Linsley. The stream connecting these two bodies of water passes along the northern edge of a golf course. Surface water flows across this well-fertilized lawn and into the stream. Twin Lakes Road runs north-south between the two lakes and storm drainage is piped directly from the road into the pond. There is a secondary inlet stream on the northern side of the pond which provides a conduit for the waters which wash the lawns and septic fields of houses on the northern slope of the drainage basin. The outflow is at the northwestern "corner" of the lake. When precipitation is heavy, the swamp on the west-northwest edge of the lake carries some of the excess water.

A variety of organisms have been reported to have produced bloom populations in Linsley in the past. At present the annual cycle is dominated by blue-green blooms which are irregularly broken by brief periods of relatively bloom-free waters and on rare occasions by spring diatom blooms, but the dinoflagellates no longer appear in any quantity. To offer background information and to provide a basis for comparison with blooms noted during this study, a table of information relating to prior blooms in Linsley Pond is included in Appendix A.

Field Trips

Field study of the pond was carried on from fall, through summer, 1974. During the first growing season, 1972, weekly field trips were made. The decision to establish a weekly sampling schedule was based on the comments of Lund and Talling (1957) and on my own past observations, both suggesting that significant, but unexpected, quantitative and qualitative changes may pass unnoticed if monthly observations are relied upon. During the later growing seasons field trips were more widely spaced; *i.e.*, 1973, every two weeks; 1974, once per month. The less precise monitoring of field conditions during the last two years was dictated by time limitations. Winter field work, 1971-1974, was on a less regular basis, 1-3 trips per month, depending on weather conditions and, especially, on the thickness of ice cover.

Sampling Station

Since preliminary work suggested very little variation in phytoplankton population levels when samples were confined to the open waters of the pond, a single sampling station at the approximate center of the pond was established. Landmarks on each of the four shores were used to properly locate the aluminum rowboat from which samples were taken. Depth measure was also used as one of the criteria for determining the precise location of the sampling station. Riley's bathymetric map of the pond (1939) indicates a fairly narrow, 14 m deep, trough running east-west along the center of the lake bottom. Depth at the station varied from 13-14 meters. This variance reflected both the changes in the actual water level of the pond and a moderate variation in the precise location of the station. No depth measure was accepted (*i.e.*, location was assumed to be inaccurate) when less than 13 m and, although Hutchinson reports a 14.8 m maximum depth in 1938, no depth greater than 14 m was recorded during the 1971-1974 sampling period.

Sampling Regime

Samples were taken at 0, 2.5, 5, 9, and 13.5 (bottom) m. During the first year of the sampling period Secchi, temperature, H_2S boundary, pH, alkalinity, hardness, D.O., and CO_2 were checked regularly. Alkalinity and CO_2 measures were done every other week; D.O. was checked on those alternate dates when alkalinity and CO_2 were omitted—a twice monthly basis for each of these parameters. From fall, 1972, onward alkalinity, CO_2, and D.O. measures were omitted from the regular sampling program. All depth samples were taken with a Meyer sampler as described by Ruttner, 1953.

VI:II PHYSICAL AND CHEMICAL: METHODS

Temperature and Dissolved Oxygen

Temperature was taken with a thermistor attached to the oxygen probe of a Precision Scientific Galvanic Cell Oxygen Analyser (D.O. meter). After D.O. measure was dropped from the sampling schedule a -20 to 110°C mercury-in-glass thermometer was employed.

Dissolved oxygen was measured every 0.5 m when the D.O. meter was employed, but was measured only at 0, 2.5, 5, 9, and 13.5 m when the Winkler method (as described in Welch, 1948) was used. The Winkler determination was employed either as a check on the accuracy of, or as a substitute for, the D.O. meter measurement. The meter was checked against a Winkler determination once every six weeks, usually when the probe required refurbishing.

D.O. samples for the Winkler determination were carefully siphoned out of the Meyer bottle and into standard B.O.D. bottles. The siphoning technique was sufficiently effective in avoiding O_2 contamination of samples to allow for zero mg/l oxygen measures to be obtained from bottom water samples.

Secchi

Although the Secchi measurement cannot be equated with light measurement in a direct or simple manner, there is some indication that the point of disappearance of a 10 cm, all white, disc approximates the depth of 5% light penetration (Hutchinson, 1957; Yoshimura, 1938); therefore, a 10 cm, all white, disc was used and values were determined according to the methods described in Welch (1948). To afford some consistency relative to the possible effects of the angle of incidence of sunlight on Secchi readings this test was done at approximately 1:00 p.m. on the day of any given field trip.

H_2S

H_2S boundary was determined by odor. Samples for this measure were taken at 0.5 m intervals beginning just above the thermocline and descending until the characteristic H_2S odor was detected.

pH

The pH of each depth sample was measured immediately at collection with Hydrion Lo-Ion, dye impregnated, pH "paper". This paper was designed for use in unusually dilute solutions such as natural fresh waters and, unlike Hydrion pH paper, produces readings which correspond closely to pH meter readings for such solutions. A second reading was made on a pH meter when samples were brought into the laboratory. Lo-Ion and meter readings were always similar. High pH values obtained with Lo-Ion paper *in situ* during midsummer afternoons usually dropped by the time samples were returned to the laboratory; however, a second Lo-Ion reading, taken in the laboratory, and the meter reading remained similar.

Alkalinity and CO_2

Alkalinity and CO_2 were measured titrametrically using the methods described in Welch (1948).

Water Samples for Biological Activity Tests

In addition to the samples for phytoplankton counts a five to twenty-five liter surface sample was taken for later laboratory assay of the biological activity of natural waters. Upon return to the laboratory this sample was first prefiltered thru fiberglass, then passed thru sterile (H.A., 0.45 μ) Millipore filtration apparatus and stored in sterile, non-linear polyethylene containers at -20° (chest freezer). According to the technical laboratory at Falcon Plastics, the presterilized 2½ liter containers employed for storage were made of a purified plastic resin which Dupont claims carried no trace metal contaminants.

On occasion a portion of this large sample was not stored, but was instead processed and assayed for biological activity immediately after collection. The bioassay procedure is described in Section VII:I. This immediate assay of biological activity was intended to serve as a comparison for those assays which were performed on the same water samples after freezing, storage and thaw. After some experience with this comparison it was determined that freshly collected samples provided more reliable information than did freezer-stored samples; therefore, assay of freezer-stored water samples was discontinued.

Weather

Ambient temperature, cloud cover, and precipitation were noted for each field trip.

Biological Oxygen Demand

The B.O.D. of samples from each of the five depths was measured twice during 1972. Measure was based on the difference between saturated D.O. levels at time zero and D.O. levels after five days incubation at 20°C in total darkness. B.O.D. bottles were immersed in water for the duration of tests to assure that no oxygen could enter and contaminate test samples. All oxygen measures were determined by the Winkler method as described in Welch (1948).

VI:III PHYSICAL AND CHEMICAL: RESULTS AND DISCUSSION

Introduction

Total alkalinity, CO_2, pH, dissolved oxygen, Secchi, H_2S horizon, thermocline (including temperature in depth), and phytoplankton population levels were monitored on a weekly basis thru the first year of this study. Data was collected for 0, 2.5, 5, 9, and 13.5 m depths to permit consideration of the significance of vertical changes. Since the first year's data indicated no unexpected perturbations in the curves generated by CO_2, alkalinity, and dissolved oxygen measures; since these tests required considerable time during any given field trip; and since indirect monitoring of these parameters is sufficient for detection of extraordinary changes; these three measures were omitted from the sampling program in the second year and thereafter. Indirect monitoring of CO_2 and alkalinity was accomplished by monitoring pH, and indirect monitoring of dissolved oxygen was possible via the monitoring of the H_2S horizon.

Dissolved CO_2

During the winter months CO_2 remained low and constant thru the water column (Figure 2). This reflected a bacterial community of moderate size and a low population of zooplankters. Thru the growing season, however, CO_2 varied with depth. The low (to zero) dissolved CO_2 in surface waters during midsummer afternoons reflected a high rate of carbon fixation and demonstrated the capacity of the blue-green bloom organisms, which consistently dominated the open waters of Linsley, to use available dissolved CO_2 before resorting to the bicarbonate ion as a carbon source.

CO_2 was always measured at approximately 3:00 p.m. On two occasions, July 18, and August 8, 1972, surface CO_2 was measured at 11:00 a.m. and again at 3:00 p.m. CO_2 levels at 11:00 a.m. were found to be 3.5 and 4.0 ppm respectively, while in both cases, surface CO_2 was 0 ppm at 3:00 p.m.

During the growing season the level of dissolved CO_2 in bottom waters reflected bacterial activity on, or near, mud surfaces. Bacterial activity increased in mid to late summer as the level of freshly deposited bottom detritus increased. The increase in dissolved CO_2 at a depth of approximately 5 m on March 28, and on October 23, 1972, probably reflects an increase in zooplankton—Keratella in March and Cyclops in October. No attempt was made at these early dates to determine numbers of zooplankton, but in both cases the zooplankters named were observed in 5 m phytoplankton counting samples.

pH

During the three years of this study the pH of Linsley Pond waters at 0, 2.5, 5, 9, and 13.5 m depths remained fairly constant (Figure 3 offers

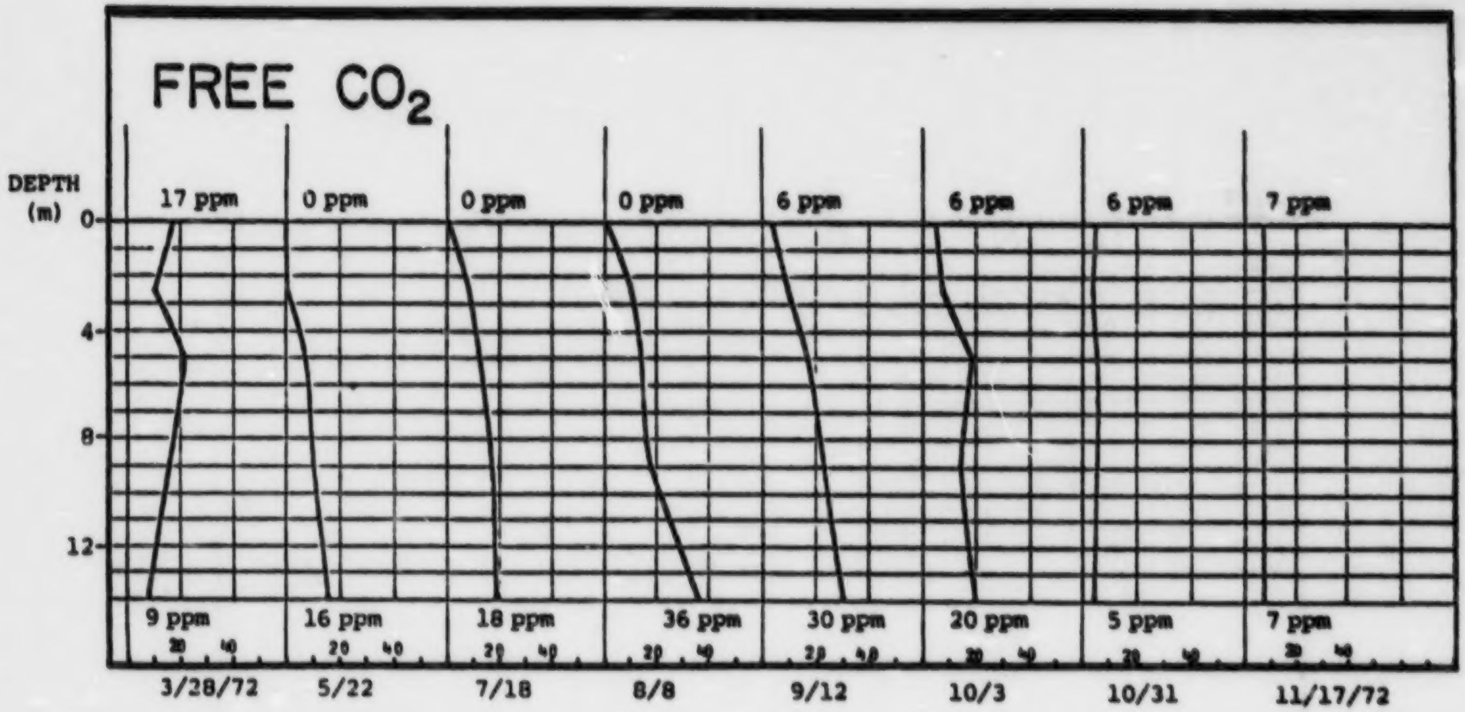


FIGURE 2. Carbon dioxide: Annual Cycle.

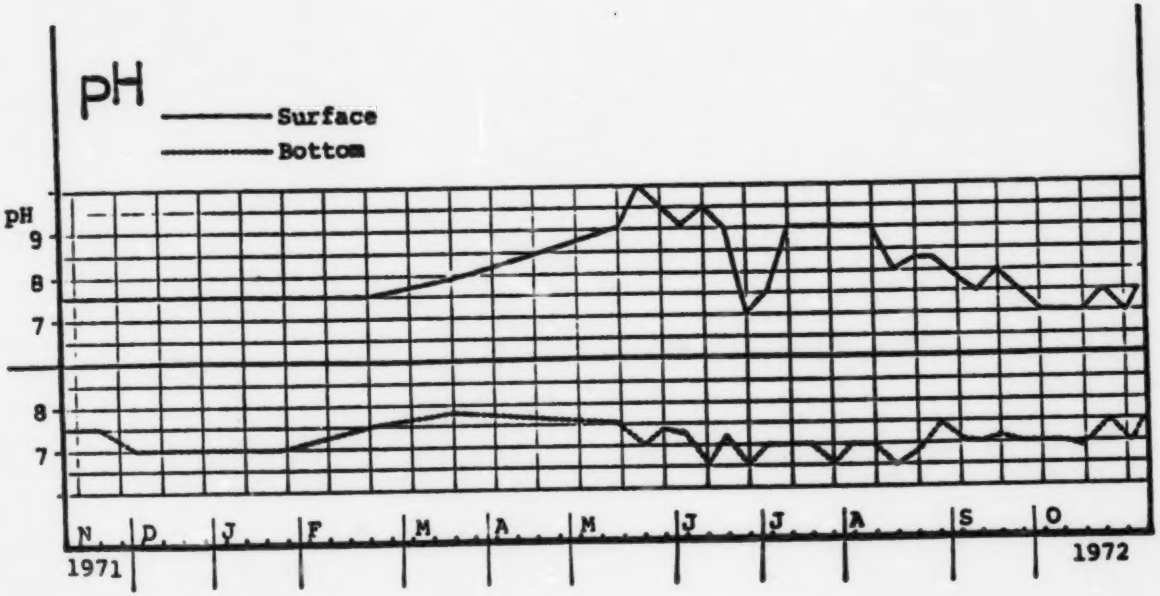


FIGURE 3. pH: Annual Cycle.

a comparison of the extremes; i.e., surface and bottom measures). The only abrupt changes noted corresponded to high productivity periods (midsummer afternoons) when dissolved CO_2 in surface waters was depleted by the high rate of carbon fixation of phytoplankton. The single low reading (7.0) in late June, 1972, reflected two unusual circumstances: first, an exceptionally heavy rainfall two days before the field trip (the radio reported a total of 12 inches) the runoff of which diluted the phytoplankton population (the period between the rainfall and the field trip was insufficient for recovery); and second, moderate cloud cover during the morning hours which limited productivity.

Through the year readings from surface and 2.5 m depths were generally similar, as were readings at 9 and 13.5 m. Five meter readings usually fell midway between surface and bottom levels except when H_2S dominated the hypolimnion, in which case pH readings for the entire hypolimnion were similar. No unexpected readings were taken during the three years of this study. A graph of the first year is provided (Figure 3) to demonstrate the annual cycle. Surface readings in general were slightly, but consistently, higher than bottom readings, reflecting both the elimination of dissolved CO_2 during periods of productivity in surface waters, and the higher organic load and decomposition rate in bottom waters and adjacent mud surfaces.

This stability reflects the natural bicarbonate buffering system of Linsley. During laboratory work dealing with the F vs A assays, a consistent return of pH values in A (autoclaved) tests to the original F (filtered) levels, usually within a week of autoclaving, also suggests a natural buffering capacity in Linsley Pond waters.

Alkalinity

No unexpected phenomena were indicated by unusual perturbations of alkalinity curves (Figure 4); however, a most interesting correlation between alkalinity measure and lake morphology in the 1970's and Hutchinson's 1938 commentary concerning this same correlation was noted.

In 1938 Hutchinson discussed the similarity between the variation of alkalinity (HCO_3^-) with depth and the morphology of the lake bottom. Using Linsley as a model, he demonstrated a correlation between the curve resulting from the graphing of the ratio of mud surface to volume and the curves resulting from the graphing of late summer alkalinity levels by depth. He noted a similarity in both first and second derivative (similar slope and points of inflection).

Thirty-five years later the same correlation can be seen between these two parameters. Although his alkalinity measurement was much more precise (measurements were taken at one meter intervals) than are the measurements of the present study (0, 2.5, 5, 9, and 13.5 m only), the resulting alkalinity in depth curve is quite similar to earlier determinations. The level of alkalinity has increased for all depths (i.e.,

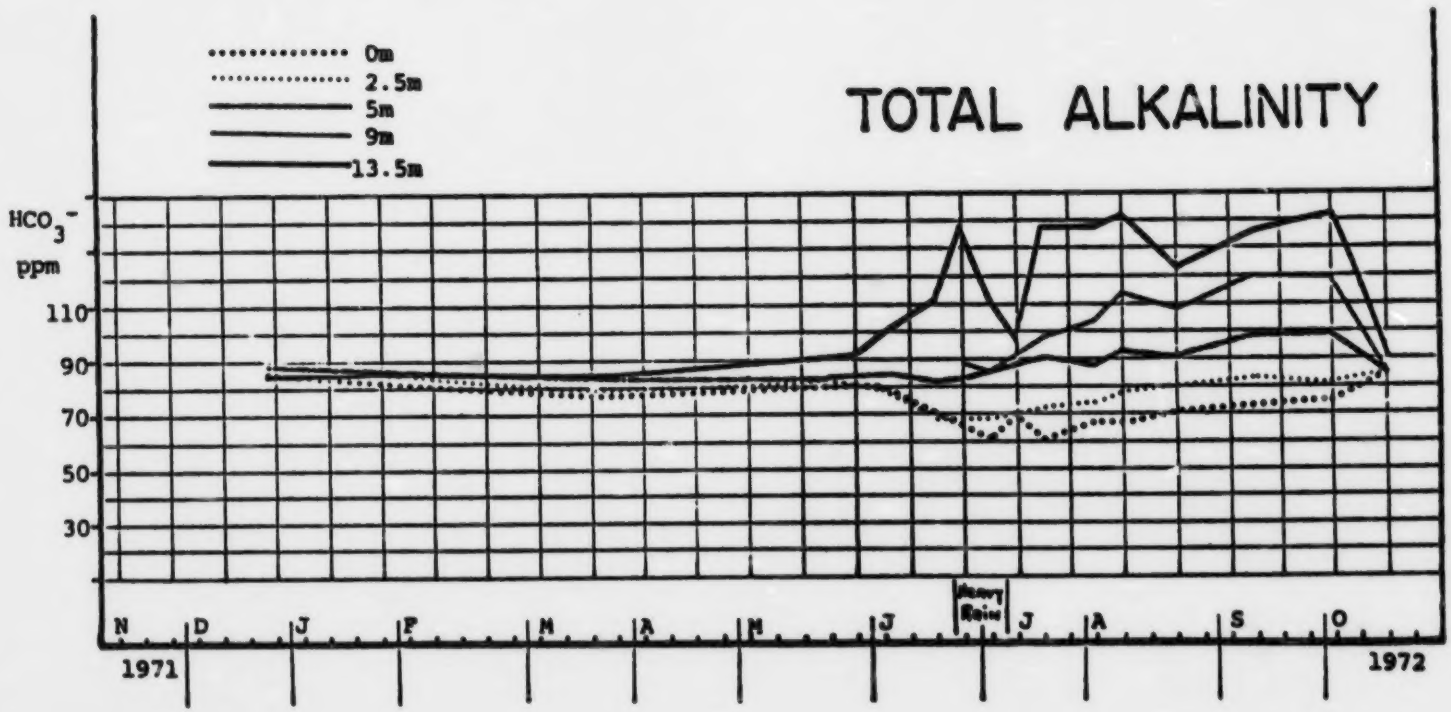


FIGURE 4. Total Alkalinity: Annual Cycle.

the curve is displaced vertically), but the slope and inflection are still similar to those of the mud surface to volume and projected mud surface to volume curves (Figure 5).

The only significant exception to this correlation is shown in the alkalinity curve for late June, 1972 when bottom alkalinity levels dropped precipitously after a period of unusually heavy rainfall and concomitant heavy runoff. This very dense runoff water, laden with the materials accumulated during its rapid flow toward the lake, may reasonably be interpreted to have entered the otherwise closed water system of the hypolimnion directly as it flowed down the surrounding hillsides, along the mud surfaces of the littoral zone, and finally, along the bottom mud of the pond.

Thermal Profile and Thermocline

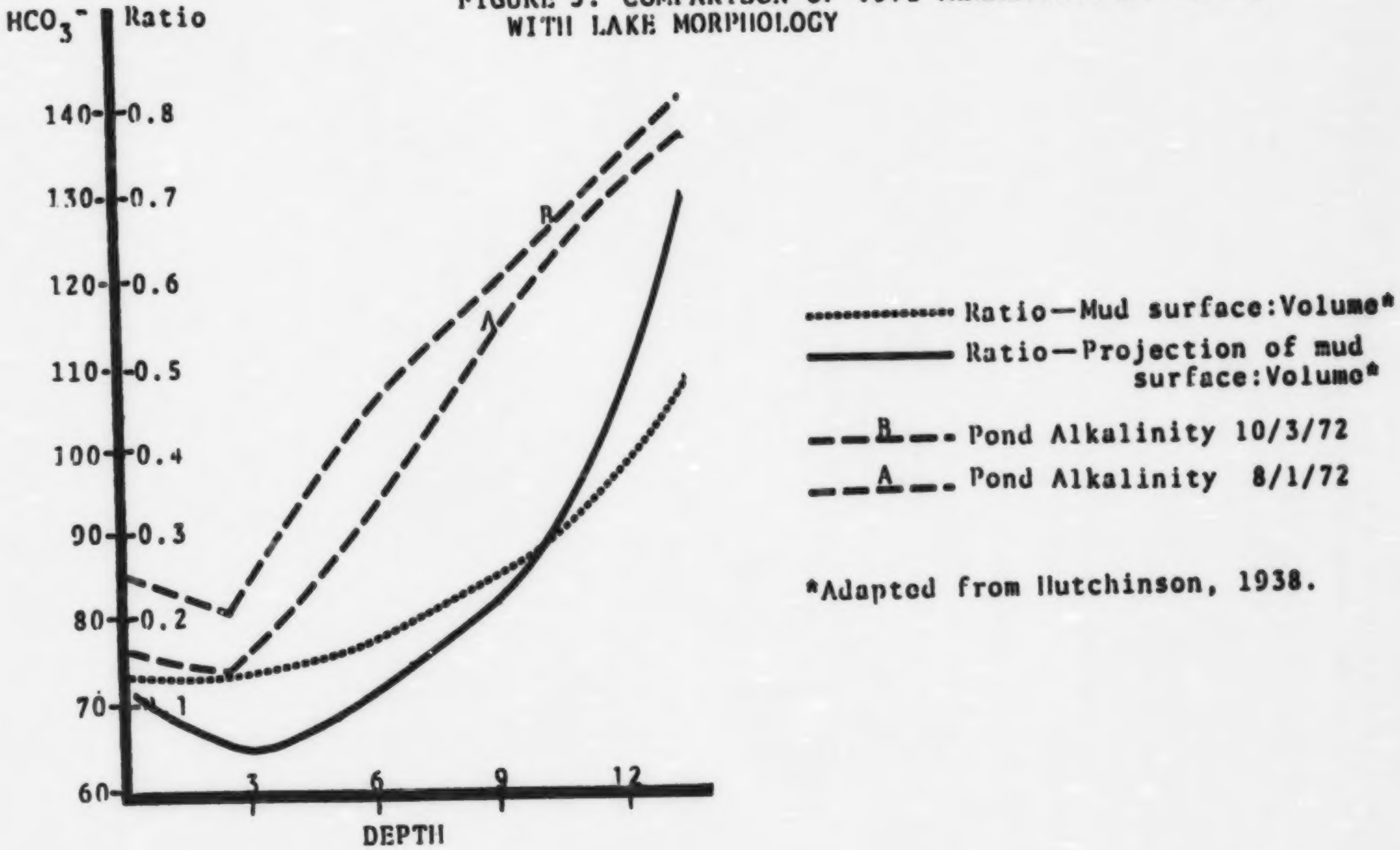
A thermal profile of Linsley was determined for every field trip. The annual cycle of Linsley's thermal regime is presented for 1972 (Figure 6). Although the profile (in depth) provided more information concerning the thermal stratification of the lake on any given date, the simple location of the approximate depth of the center of the thermocline (Figure 7) when plotted thru the entire study period offered a more informative picture of the water dynamics resulting from the thermal cycle. This representation of the vertical movement of the thermocline permits comparison not only of the timing and length of overturns, but also of the long stable stratifications of summer waters, and of the relatively brief, less stable, winter stratification periods—during which the entire body of lake water was very close to a single temperature, extremes being no more than 4°C apart.

Dissolved Oxygen and H_2S Horizon

As with previously discussed parameters, dissolved oxygen profiles generated by the data of this study represented those to be expected for a medium-sized, eutrophied, lake such as Linsley (Figure 8). In less productive winter and spring waters the clinograde oxygen distribution common to eutrophied lakes occurs. In the highly productive summer months, however, the total elimination of dissolved oxygen from the hypolimnion produced an orthograde curve with the appropriate absolutely uniform vertical distribution of oxygen (albeit at zero).

Like a thermal profile (in depth), an oxygen profile can offer a clear description of the condition of lake waters only on a single occasion. But the simple location of the zero D.O. boundary, when plotted against time, can provide additional meaningful information relating to the annual cycle of the dynamics of the putrefication process which diminishes the aesthetic acceptability of highly productive waters. In Linsley the zero D.O. depth was always a close approximation of the H_2S horizon (Figure 9).

FIGURE 5. COMPARISON OF 1972 ALKALINITY (BY DEPTH) WITH LAKE MORPHOLOGY



*Adapted from Hutchinson, 1938.

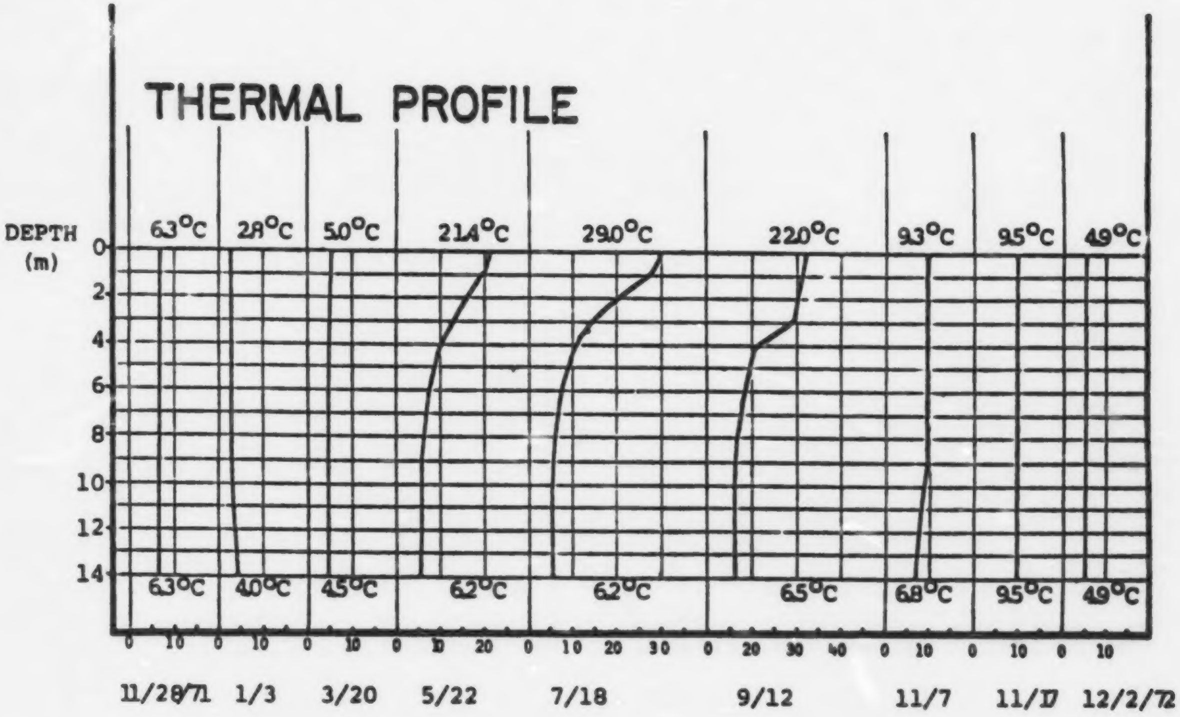


FIGURE 6, Thermal Profile: Annual Cycle.

THERMOCLINE

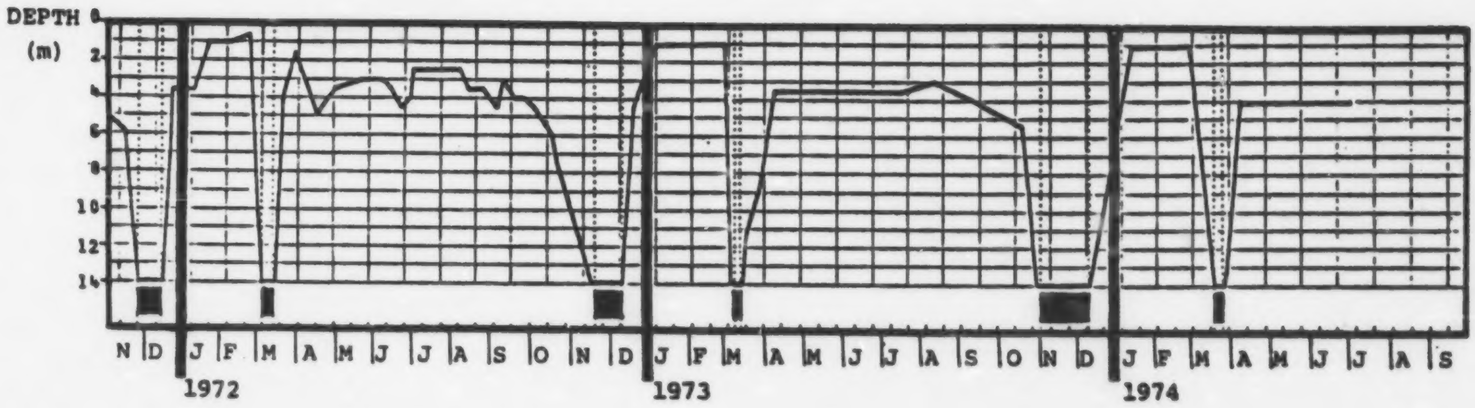


FIGURE 7. Depth of Thermocline. Three Years.
Spring and Fall Overturns indicated in Bottom
Margin by Solid Black Bar.

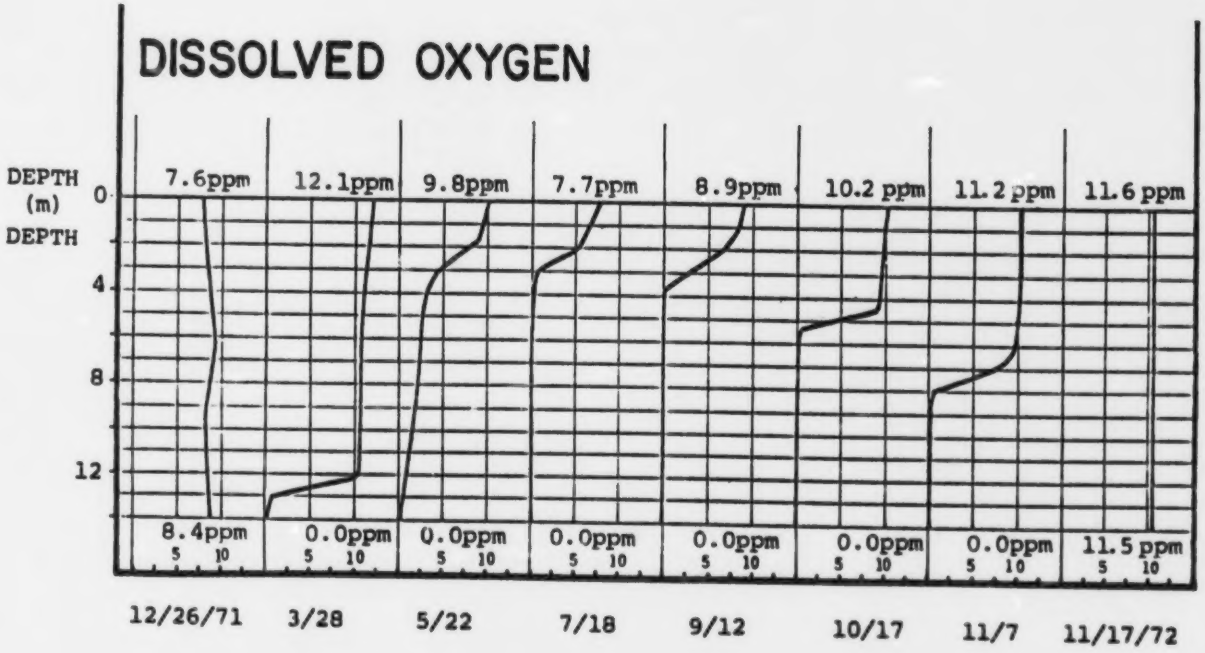


FIGURE 8. Dissolved Oxygen: Annual Cycle.

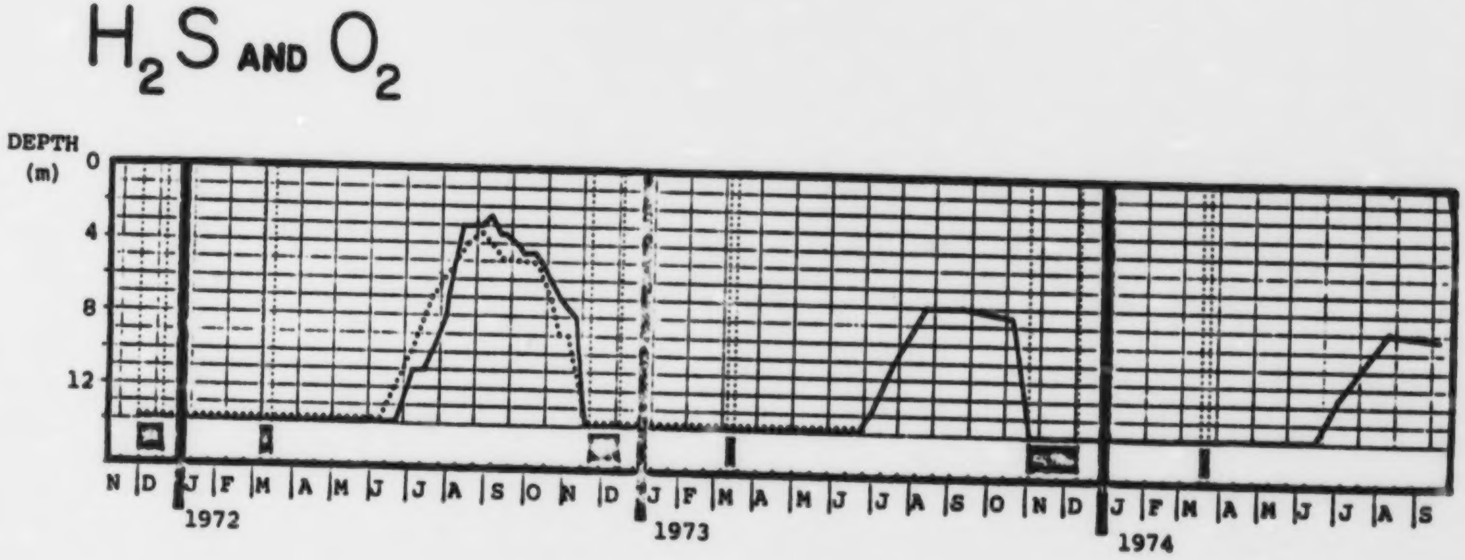


FIGURE 9. Hydrogen Sulfide Horizon: Three Years.
Spring and Fall Overturns Indicated in Bottom Margin by Solid Black Bar.
D. O. = Zero Line Included for Comparison: One Year.

Some minor discrepancy between these two parameters appears in the graph in Figure 9. Although this may be interpreted as the result of very limited mixing at the interface of oxygenated and H_2S dominated waters, I believe it to be an artifact of measuring techniques which required sampling for H_2S and testing for D.O. to be temporally, and therefore, almost certainly, spatially, separated. This latter explanation is additionally favored if one considers the capacity of modules of lake water to be in constant motion, not only in relation to the sampling station (boat), but also in relation to each other. Also, the "mixing" explanation could only be applied to those occasions which show overlapping of the two regions. It could not be applied to those occasions wherein the two parameters do not touch.

Evidence of the changing conditions in Linsley waters during this study is found in the three year "H_2S Boundary" graph (Figure 9). That the 14 m deep basin which holds Linsley's waters was filled with H_2S laden waters to within 2.5 m of its surface in the summer of 1972, but carried at least 6 m of H_2S-free water during midsummer, 1973 (doubling the depth of acceptable water), and improved again during midsummer, 1974, is a clear demonstration of the slow improvement in the condition of Linsley's waters. This is, unfortunately, less improvement than would have been assumed based on the visual and tactile judgements which accompany recreational contact; however, this contrast in conclusions drawn from the subjective recreational contact and the objective monitoring of H_2S boundary attests to the value of H_2S boundary data as a rapid measure of bloom productivity.

Secchi

Among the tools of science, the Secchi disc is unique. It is highly valued for its consistent performance—yet its measurements of a physical quantity must be expressed with a unit which describes length, and which has only relative meaning when used for Secchi readings. Yoshimura (1938) may have been accurate in his contention that a 10 cm, all-white, Secchi (as used in this study) disappears at the depth at which light is approximately 5% of the ambient light level; there are, however, a variety of patterns and sizes of Secchi discs. With, or without, a tie-in to some physically defined unit, the Secchi can provide a general measure of conditions in any given body of water; and a few notes relative to the color, opacity, dominant plankton, and detritus taken at time of the Secchi reading provide an excellent, very rapid, assessment of such conditions.

Any single Secchi reading offered an immediate basis for the description of Linsley as highly eutrophied; however, there was little additional input from a single measure if taken by itself. In this study the greatest value of a Secchi measure (Figure 10) was its relative similarity, or difference, to each of the other Secchi measures of the study. During a bloom period a rise or fall of 10 to 20 cm in the Secchi depth provided factual input for an immediate in situ decision as to whether a

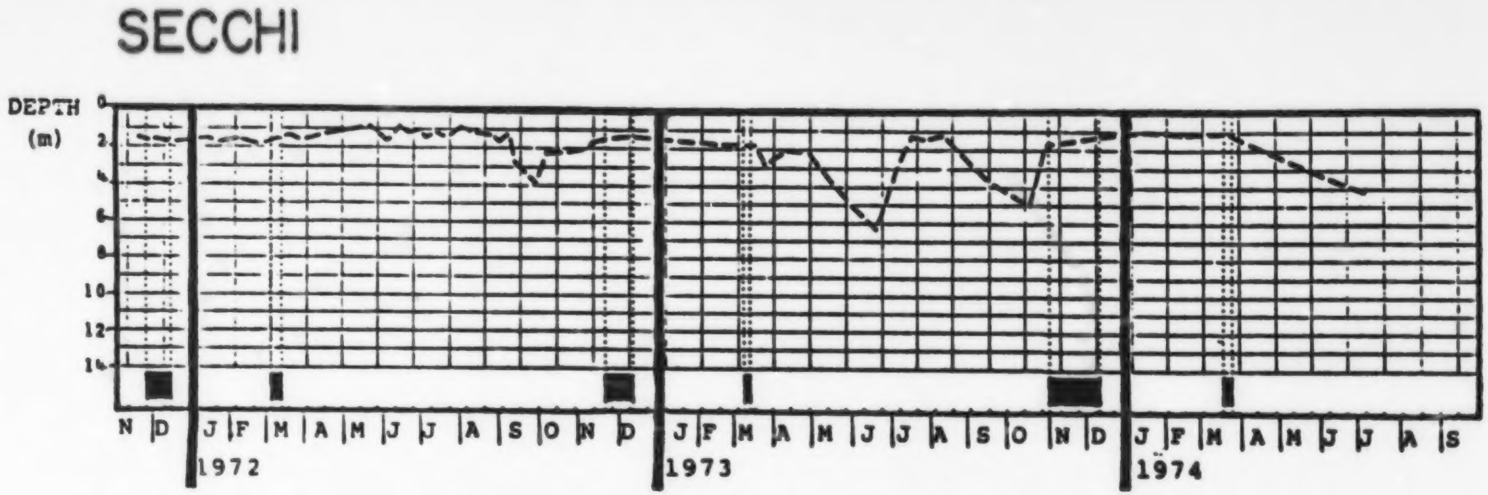


FIGURE 10. Secchi Depth: Three Years.
Spring and Fall Overturns indicated in Bottom Margin
by Solid Black Bar.

specific week marked the beginning, or the end of a bloom; or whether the phytoplankton community was similar to that of the previous sampling. Without the input from the Secchi such judgements would have been based on visual memories of the appearance of samples taken during a previous field trip, and this would have been the only basis for such judgements until several hours later when time-consuming population counts were completed back in the laboratory.

Thus, the weekly progression of the Secchi depth (Figure 10) proved especially valuable in the field. The obvious difference between 1972 and 1973 summer readings provided immediate assurance at the start of each field trip that Linsley was markedly clearer in 1973 than it was in 1972. Later in the study similar comparisons indicated that conditions in 1974 were similar to those of 1973. These facts would not have been immediately obvious during field trips if only the general appearance of surface samples was considered. For example, the plankton in surface samples was not as dense in 1974 as it had been in 1973. This gave the erroneous impression that waters were less dominated by algal blooms. Actually, the 1973 bloom was a surface phenomenon, and the 1974 bloom was a subsurface phenomenon, both populations had similar effects on the overall quality of the water.

Biological Oxygen Demand

The biological oxygen demand of 0, 2.5, 5, and 13.5 m water samples was measured on February 23, 1972 and on August 8, 1972 (Figure 11). Results indicated that Linsley B.O.D. levels were within the range of those from other moderately eutrophied lakes. When compared to the levels of as high as 50 mg per liter in secondary sewage effluents, Linsley values were low. When compared to the almost zero levels of oligotrophic mountain waters, however, Linsley values were moderately high.

General Discussion of Physical and Chemical Results

The physical and chemical parameters monitored during the study were found to be within the ranges expected for a lake of Linsley's size and degree of eutrophication. The improvement in the condition of the lake from the summer of 1972 to the summers of 1973 and 1974 is most readily demonstrated by the increase in Secchi depth and by the decrease in the volume of H_2S dominated waters. It is probable that this improvement is the result of the correction of a single faulty septic system. Raw sewage was being diverted directly into the pond via an outlet stream on the northeastern boundary of the lake. Corrective measures were apparently made only in response to the threat of legal action against the offending property owner. There is a possibility that at present sewage is again being diverted into the pond because the endemic Oscillatoria rubescens (535) population has produced its first bloom since the winter of 1971-1972, and that bloom was supported by the high nutrient level during the time period when raw sewage was entering the pond.

B.O.D.

DEPTH (m)	0	2.5	5	13.5
Feb. 23, 1972	3	3	2.5	6.5
Aug. 8, 1972	7	7	6.5	5

FIGURE 11. Biological Oxygen Demand—5 Days. Expressed as the Difference between D.O. ppm (Saturated) on Day 0 and D.O. ppm on Day 5.

The original determination of sewage in the inflow was made by an engineering firm. They reported very high (10^5/ml) Escherichia coli counts in the stream. After correction, the E. coli count was very low (10^2/ml). Periodic check on this particular stream is now planned by members of the Linsley Pond Association to assure the permanent compliance of the offending party with the legal requirements regarding his septic system.

VI:IV BLOOM SEQUENCE MONITORING: METHODS

Phytoplankton

Samples for phytoplankton counts were taken at 0, 2.5, 5, 9, and 13.5 m. 200 ml portions were stored in glass bottles which were, in turn, placed into individual styrene thermal containers. Phytoplankton counts were made immediately after return to the laboratory on the day of any given field trip. Preserving specimens for later counting was not considered satisfactory because many forms, especially the prokaryotes, were distorted or destroyed by the chemical action of preservatives and this distortion, or destruction, was considered to interfere with accurate counting and species recognition.

The majority of counts were made at 400x and 1000x (oil) on a standard Zeiss microscope using a Modified Neubauer "Brightline" Hemacytometer. Exceptionally small organisms, for example the Synechecoccus sp. (91) which occurred in significant numbers in the spring of 1973, were counted at 520x and 1000x (oil) with the aid of the interference phase contrast and dark field equipment of a Galileo L.G. 5 microscope. Counts of the Synechecoccus sp. (91) were 400-500% higher when the more sophisticated equipment was employed. This discrepancy reflects the generally small size and low pigment density of most prokaryotic forms.

A Sedgewich-Rafter counting chamber was used for counting larger forms, especially the Anabaena sp. (765) which dominated during the summer of 1973. Counts of larger organisms were also made by passing a 200 ml sample thru a Millipore (H.A., 0.45μ) filter with grid markings, and counting organisms at 100x and 200x with the aid of a Leitz inverted scope. This filtration method worked well only for very large forms such as the aforementioned Anabaena sp. (765), Coelosphaerum, or Eudorina, and for more rugged forms such as Trachelmonas and Ceratium. Volvox colonies, in contrast, were frequently unrecognizable after filtration and were, therefore, counted only in the Sedgewich-Rafter chamber.

Although the standard is somewhat arbitrary, 100 organisms were considered to be the standard counting sample. This means that from 5 to 20 separate sample drops were utilized in most of the counting procedures. This standard was held only for major constituents of the population. Organisms present at 1000/ml or less were represented by fewer individuals for practical reasons. The 100 cell count is suggested by Lund and Talling (1957) as a reliable numerical sampling of individual organisms in population counts.

Brook, et al. (1971), used a measure of turbidity to approximate population density for *Oscillatoria agardhii* and were satisfied that they were measuring the correct parameter with the turbidimeter. On several occasions, parallelling this method, an O.D. meter which accepts test tubes (made for Haskins Laboratory by Dr. Fred Kavanaugh)* was used to obtain population profiles in depth of *Oscillatoria rubescens* (a form of *O. agardhii* which carries sufficient phycoerythrin to give a bloom a reddish-brown, or orange, tint). The correspondence of O.D. to counts was fairly good; however, maximum populations produced O.D. values in the lowest part (least reliable) of the O.D. meter range and the possibility of inaccuracy was considered too great to permit the substitution of O.D. for direct counting.

Zooplankton

Monitoring of zooplankton populations was not one of the goals of the original proposal for this study. Zooplankton were specifically omitted: first, because preliminary survey indicated very few zooplankters in the open waters of Linsley Pond; and second, because the mechanics of collecting even a moderately accurate numerical sampling of zooplankton are cumbersome and, as with all collection techniques, become increasingly more complex as the density of the population to be sampled drops.

Incidental data relating to zooplankton counts, however, proved to be of such interest that it would be a greater error to ignore it than to include it, even while conscious of its weaknesses. The quantitative aspects of zooplankton population monitoring in this study are only meaningful in judgements concerning relative changes and cannot, therefore, be considered as measures of absolute numbers.

Zooplankton counts were made by passing approximately 200 ml of surface water samples (collected by dipping and scooping water into jars) thru a (H.A., 0.45μ) Millipore filter with grid markings. This "counting" of zooplankton was prompted by the dramatic appearance of zooplankton in great numbers early in the 1973 growing season. In the Fall of 1971 and thru the growing season of 1972 it was common to pass as much as 10 liters of surface pond water (collected for purposes of storage for later bioassay) thru a large fiberglass filter (held in a Buchner funnel) and to note no zooplankton in the "concentrate" above the filter. Some zooplankters are known to actively avoid the suction current caused by submerging a container; however, these large samples in 1972 usually produced no zooplankton whatsoever (curiosity, prodded by this total absence, occasionally prompted careful search). In contrast in 1973 these same large samples, taken with the same containers, in the same fashion, evidenced hundreds, at times thousands, of zooplankters.

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*See Appendix D for additional information re this meter.

In addition, because I have the Ceriodaphnia and Eubosmina from Linsley in culture at the present; and because (although I have repeatedly failed in attempts to initiate cultures of Cyclops, Keratella, and the rather rarely noted Calinoid cyclopod from Linsley) I have had considerable experience in attempting to catch, and ample opportunity observing, the zooplankters of Linsley; I have noted that while Cyclops and the Calinoid are very active in avoiding suction currents, the Eubosmina, Ceriodaphnia and Keratella are not.

Considering all the above facts, it is at least certain that the 1972 zooplankton population was extremely low in number, and that the 1973 population was dramatically higher.

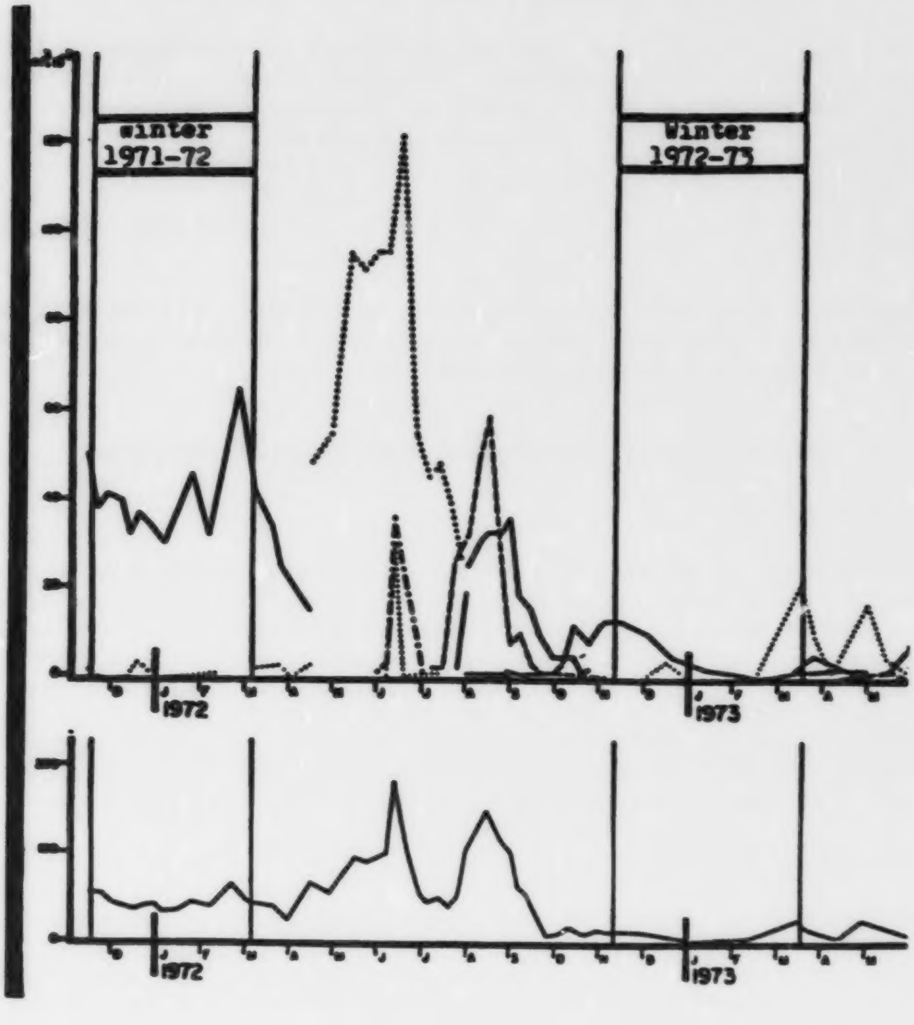
VI:V BLOOM SEQUENCE MONITORING: RESULTS AND DISCUSSION

Introduction

The phytoplankton bloom sequence of Linsley Pond was monitored for three years (winter, 1971 thru fall, 1974—Figure 12). The pond flora was dominated by an almost continuous series of blue-green blooms. Brief occurrences of diatom blooms in the springs of 1973 and 1974 were the only occasions during this study when bloom dominants were not blue-green algae. Figure 12 is offered to allow a general view of the three years of the study; however, detail of bloom sequence events is more readily gleaned from a study of the enlarged segments of this graph which are included within the discussions of each seasonal segment (key on page 54).

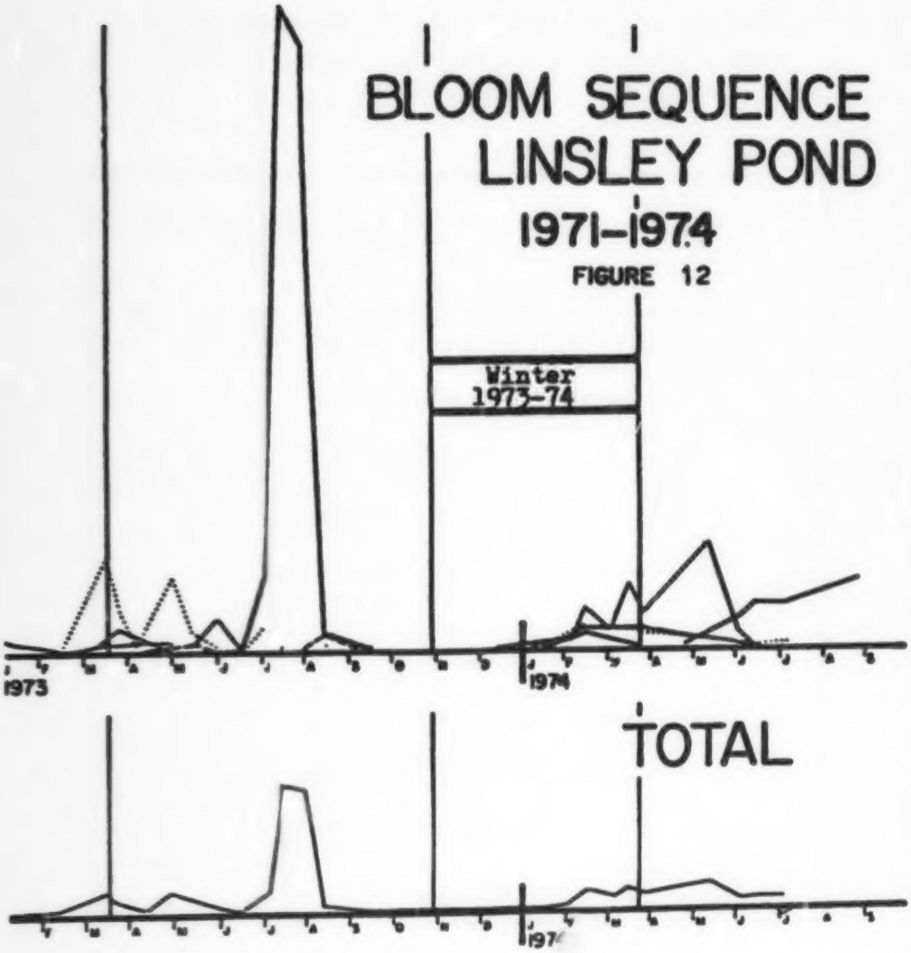
1971-1972, Winter

Thru the first winter of this study (Figure 13) the open waters of Linsley were dominated by a bloom of Oscillatoria rubescens (535). The population level which prevailed throughout the cold months was established prior to turnover. A preliminary field trip to Linsley in the fall of 1972 indicated that, if the total population of O. rubescens (535) can be approximated from surface to 11 meter observations only (counting samples were taken from irregular sampling stations—this was prior to the establishment of a single, lake-center, station), then the population level which prevailed throughout the cold months was established prior to turnover. This point is important in the discussion of a possible approach to control of Linsley's eutrophication problems by the use of biological and chemical controls to program the bloom sequence in situ, see Section IV.



Volume expressed as mm^3 x 10^{-3}

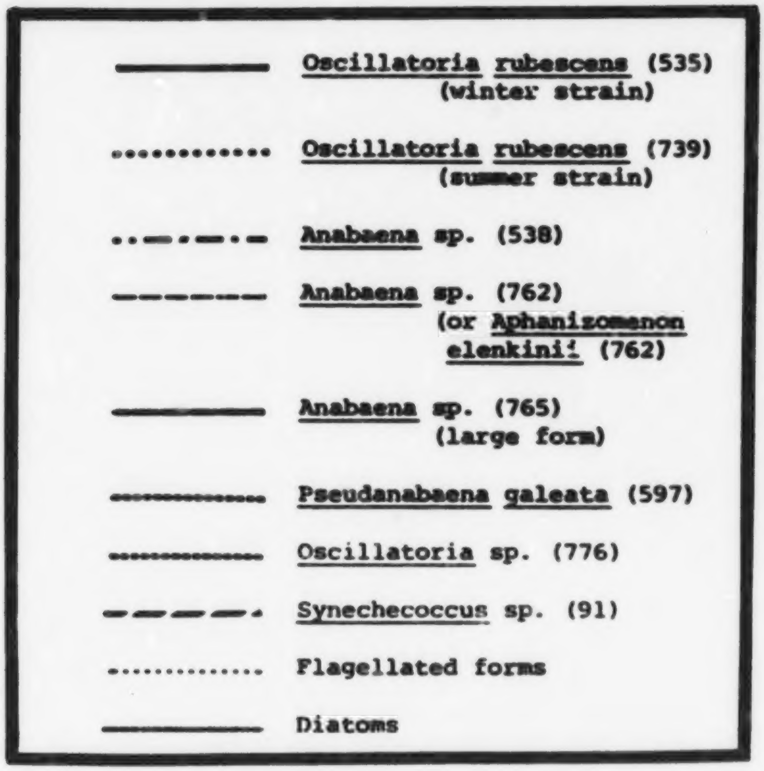
Figure 12. Key to Symbols on page 54.



BLOOM SEQUENCE
LINSLEY POND
1971-1974

FIGURE 12

FIGURE 12. Phytoplankton Bloom Sequence of Linsley Pond. Winter, 1971 to Fall, 1974.


Oscillatoria rubescens (535) (winter strain)
Oscillatoria rubescens (739) (summer strain)
Anabaena sp. (538)
Anabaena sp. (762) (or Aphanizomenon elenkinii (762)
Anabaena sp. (765) (large form)
Pseudanabaena galeata (597)
Oscillatoria sp. (776)
Synechecoccus sp. (91)
Flagellated forms
Diatoms

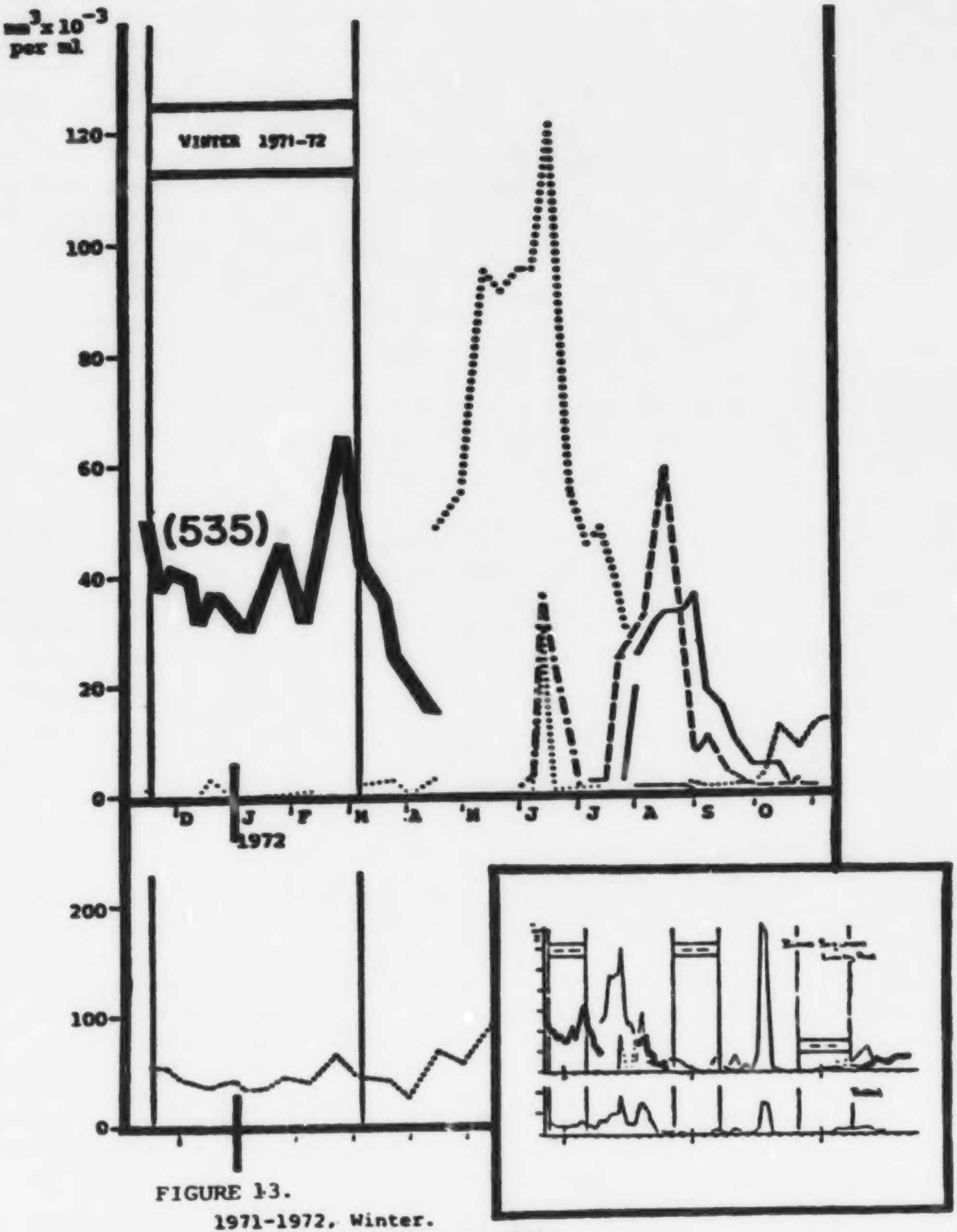


FIGURE 13.

1971-1972, Winter.

Oscillatoria rubescens (535).

Inset indicates other occurrences of *O. rubescens* (535).

Distinguishing Winter and Summer Forms of *Oscillatoria rubescens*

Oscillatoria rubescens (535) is a filamentous, non-heterocystous, blue-green algae which exhibits a positive phototrophic response both *in situ* and *in vitro*. This phototrophism, and several additional characteristics, serve to distinguish the winter strain, (535), from the summer strain, (739), of *O. rubescens*. The cell height to cell diameter ratio in *O. rubescens* (535) is less than or equal to, one—while that of *O. rubescens* (739) is greater than, or equal to, one. Additionally, different growth characteristics on agar—colony form, color, ease of establishment—distinguish the two strains. The colors of liquid cultures also provide points of difference. A healthy, liquid culture of *O. rubescens* (535) is a dark green-brown to khaki. A pink to light Winkler "peach" color appears in liquid cultures of *O. rubescens* (535) in late senescence. In contrast, healthy, liquid cultures of *O. rubescens* (739), the summer dominant, appear green-brown to orange-brown and develop a white-orange hue in late senescence. Cultures on agar maintain colors identical to the colors of healthy liquid cultures of the same strain with only a slight change toward the colors of senescence. Agar cultures dry out before they reach senescence. They maintain vitality considerably longer than do liquid cultures; some have survived thru as long as nine months. Only the drying and separating of the agar ended these cultures.

The summer form, (739), produces growth on agar which resembles thinly stacked filaments, patterned in long, thin, sweeping curves. Eventually filaments are singly and evenly dispersed thru the entire volume of the agar—suggesting an inherent capacity for motility with no concomitant phototrophic response. The winter form, (535) however, which is difficult to establish in culture on agar (many attempts produced only one successful culture), does not produce thin, sweeping curves, but is rather made up of thick, slightly curving, colonies of parallel filaments. These colonies, less definable as to shape, but a consistent dark green-brown color, do not spread thru the volume of the agar. Instead they remain on the surface, in apparently good condition, until the agar dries (sole example: five months).

Neither of these *Oscillatoria rubescens* strains has reproduced in the absence of its accompanying bacterial community. In both cases, single, axenic, trichomes have been isolated from agar and placed in test tubes. Sterility tests have shown these "cultures" to be bacteria-free. Trichomes appear intact and viable after several months, yet no apparent growth, reproduction, or disintegration occurs. The result of this apparent dependence on some bacterial metabolite, or process, is that both strains are represented in the Linsley culture collection as bacterized isolates only. The addition of filter-sterilized media, collected from a bacterized, healthy culture of *O. rubescens* might provide a successful axenic culture of these organisms. This procedure could not be included in this study, but offers promise for future work.

Oscillatoria rubescens (535), winter dominant, was successfully cultured in liquid from an opaque, red, plastic container of stored pond water (5°C in a darkened cold room). The inoculum for this culture was taken from the stored water after approximately four weeks in storage and although growth, or reproduction, during this four-week period cannot be documented, the organisms obviously survived and maintained (or improved) vitality while in total darkness—an indication of heterotrophic tendencies. This, too, offers opportunity for future study; i.e., the investigation of the heterotrophic nature of these blue-green aerobic (possibly also anaerobic - see Figure 21, this section) prokaryotes.

Oscillatoria rubescens as Opposed to O. agardhii as the Proper Taxonomic Placement for the 1971-1972 Winter-Summer Dominant Blue-Green Organism—To avoid a misinterpretation of the appearance of Linsley Pond during blooms this separate section has been included. The two dominant species of Oscillatoria; i.e., (535) and (739), were designated as O. rubescens, rather than as O. agardhii, solely on the basis of the visible presence of phycoerythrin; however. this pigment is not sufficiently concentrated to cause Linsley to turn a blood red color, as do the European lakes dominated by blooms of O. rubescens. Linsley takes on only an orange-tan color during its extremely dense blooms. Thus, these two O. rubescens strains, while qualifying technically as members of the species, do not exhibit the most infamous characteristic of the type.

1972, Spring

In the spring of 1972 the O. rubescens (535) population was replaced by O. rubescens (739), summer form (Figure 14). The breaking and deterioration of (535) was accompanied by the appearance of new healthy filaments of (739). The graph in Figure 12 indicates an additional reason for separating these two similar organisms into distinct, overlapping populations; it clearly shows two distinct population peaks (February and June).

One of the previously listed bases for this separation of (535) and (739) into two distinct strains, the phototrophic response peculiar to (535), is displayed by a graph plotting the vertical location in the pond waters of the bulk of the bloom population (Figure 15). In contrast to the apparently erratic location in depth of the bulk of the (535) population thru the winter, note the constant depth (at 2.5 m) of the bulk of the (739) population thru the spring, and its relatively constant location in, or near, surface waters in early summer.

The location in depth of the O. rubescens (535) winter bloom was dependent on the phototrophic response of (535); i.e., on a very dark (heavy overcast) winter day the bulk of the bloom would remain near the lake bottom (an early morning check in January, 1972, suggested that the bulk of this population was near the bottom of the lake each morning) while on a very bright day the bulk of the population would be near the surface. It was

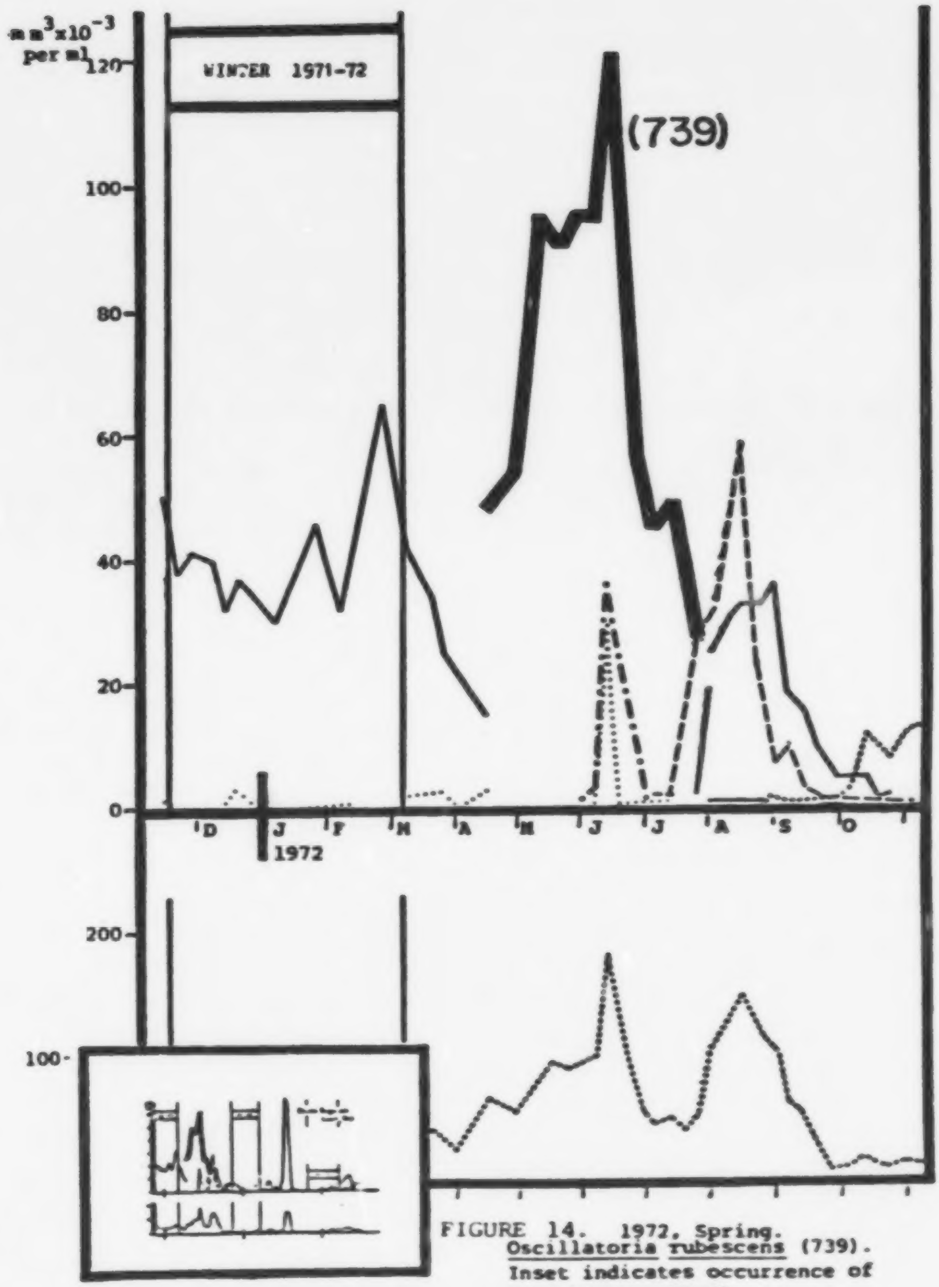


FIGURE 14. 1972, Spring.
Oscillatoria rubescens (739).
Inset indicates occurrence of
O. rubescens (739).

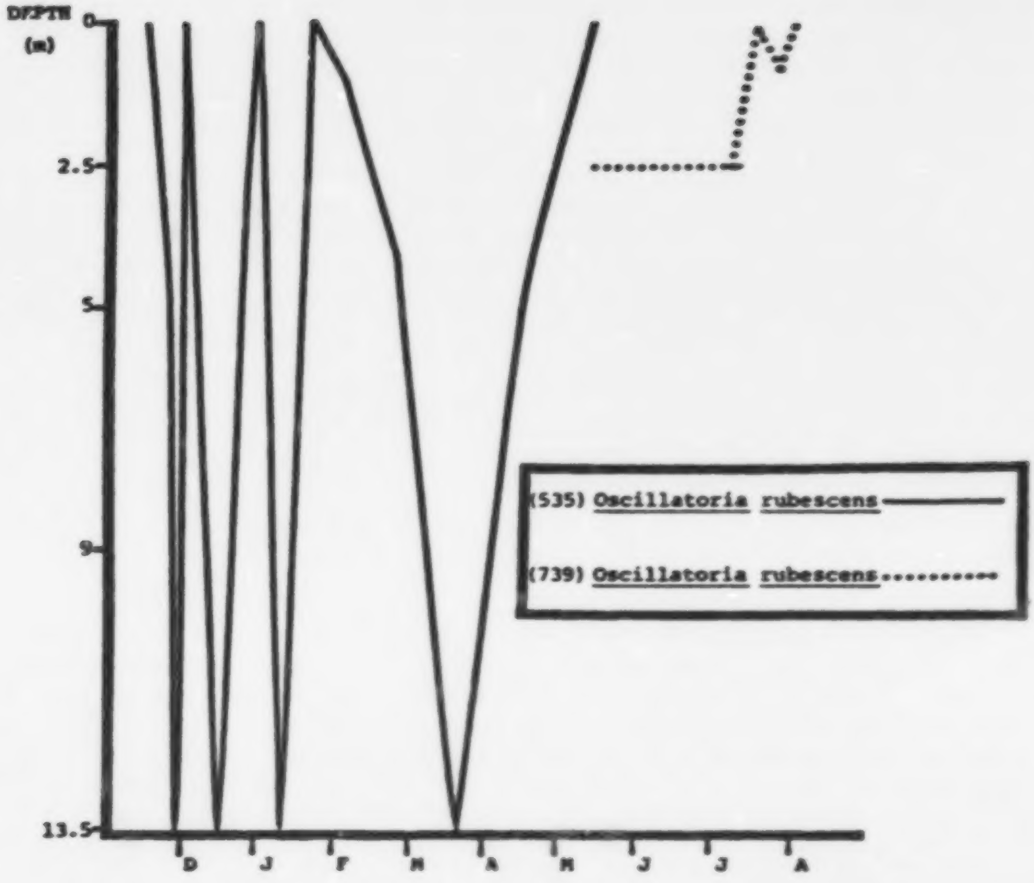


FIGURE 15. Depth of *Oscillatoria rubescens* (535) and (739) bloom populations in the lake-for comparison.

essential to determine light conditions for the entire morning of a given field trip, rather than simply light conditions "at collection", in order to predict the depth at which the (535) population would be found; however, with a little practice prediction became quite accurate. In large filtrate cultures (535) was observed to migrate to the brighter side (still relatively low illumination) of the flask. (739) did not exhibit any form of photokinesis.

During the 1971-1972 winter-spring time period many other organisms were noted from week to week. None appeared in quantity sufficient to be of significance when compared to the volume of the *O. rubescens* blooms. It was, nevertheless, during this period when most of the organisms represented by cultures in the Linsley collection were obtained.

Cyclotella sp. (211) appeared in the cold surface waters of the pond in January and, as Gordon Riley (1940) suggested, was apparently not native to Linsley but was rather washed down annually from Cedar Lake. Since the *Anabaena sp.* (538), which produced a heavy bloom in late spring, 1972, produces a heat-labile substance which is toxic to *Cyclotella sp.* (211), one may conclude that the inability of this organism to establish a permanent (if small) population in Linsley is the result of its inability to coexist with at least one of the bloom producing organisms of the pond.

1972, Late Spring and Summer

In the late spring of 1972 *Anabaena sp.* (538) produced a bloom population which became co-dominant with that of *O. rubescens* (739) (Figure 16). Both the *Anabaena sp.* (538) and the *O. rubescens* (739) blooms had subsided by the end of July, 1972. At that time two significant forms, morphologically similar to (538) and (739), replaced them as dominants. The first was *O. rubescens* (535), the winter dominant. Its population developed rapidly, reaching levels in August similar to those of the previous fall, 1971. The second significant organism was actually a combination of a few single (young colonies) filaments of *Aphanizomenon flos-aquae* (766), and a large population of a second heterocystous blue-green (762) which is tentatively identified as either *Anabaena sp.* (762), or as *Aphanizomenon elenkinii* (762). It is of interest that the *in situ* appearance of (762) was very similar to that of the spring bloom dominant, *Anabaena sp.* (538). It can be distinguished from (538) by the more conical shape of its terminal cell (a significant feature of the genus *Aphanizomenon*) but its heterocysts appear very similar to those of (538). It does not form flakes.

With the *O. rubescens* (535) and *Anabaena sp.* (762) bloom populations came three additional, non-dominant, heterocystous, filamentous, blue-green forms: *Anabaena sp.* (765), sparsely distributed, of unusually large size—ranging up to 2mm long; *Anabaena circinalis* (769), similar in size to (762) and (538) but with a very different heterocyst pattern (even interspacing with heterocysts often in pairs); and the aforementioned

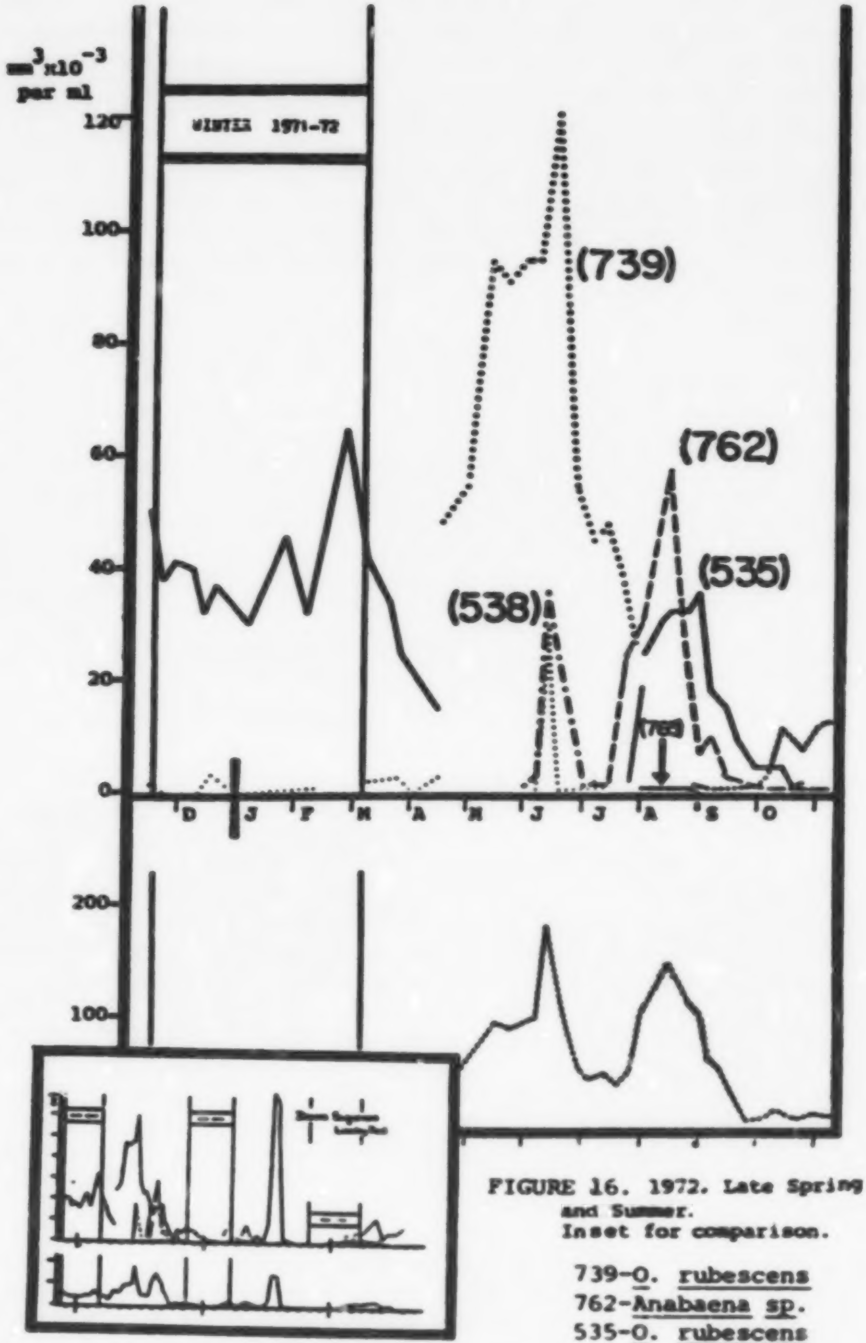


FIGURE 16. 1972. Late Spring and Summer.
Inset for comparison.

739-O. rubescens
762-Anabaena sp.
535-O. rubescens
538-Anabaena sp.

Aphanizomenon flos-aquae (766) in its readily identifiable flake form. None of these were represented by large populations in 1972. Significantly in 1973 when the spring bloom of (538) was absent, and when Synechecoccus sp. (91) produced an early spring bloom Anabaena sp. (765) bloomed. Filtrate studies indicate that (91) produces an enhancer for, and (538) produces an inhibitor for, Anabaena sp. (765).

In September, 1972, the second appearance of O. rubescens (535) (the dominant of the previous winter) ended abruptly, as did those of (762), (765), (766), and (769). This sudden ending, which also occurred in September, 1973, does not seem to correlate with unexpected physical or chemical changes in the pond; however, monitoring trace metals proved impractical, and a sudden, if minor, change in the level of these or some other critical nutrient might account for the sudden, generalized population drop. Cowgill (1971), in her comprehensive study of the elements in Linsley water (by depth and by date, weekly), found ample supplies of nutrients in August. Unfortunately, her study ends at the beginning of September and, therefore, can offer no assurance that the August levels were carried over into the next month. Tests of pond water samples from late August, 1973, when an extensive bloom of (765) ended, indicated no F vs A difference in the growth of Anabaena sp. (765) and no appreciable improvement in growth when waters were charcoal treated to remove organics. This suggests nutrient limitation rather than allelopathic effects.

1972-1973, Fall and Winter

In late September, 1972 the waters of Linsley were relatively free of phytoplankton (Figure 17). In early September a blue-green, Pseudanabaena galeata (597), which had appeared sporadically throughout the prior year, appeared in quantity. By mid October it had produced a bloom. In November this bloom was replaced by Oscillatoria sp. (776). This bloom, (776), waned slowly. It was not totally eliminated until the spring of 1973. These organisms cannot easily be distinguished during counting procedures; however, by filtering a large sample (one liter or more) the color of the dominant organism can be determined. Pseudanabaena galeata (597) is a vivid blue-green color, Oscillatoria sp. (776) is green-brown to orange-brown. Neither of these latter blooms, (597) or (776), represented the same, or even similar, volume as the bloom of O. rubescens (535) during the first (1971-1972) winter. The waters of Linsley, as can be seen by comparing the maxima and duration of 1971-1972 and 1972-1973 winter blooms (Figure 12), were considerably free of blue-green algae the second winter than they were the first. This is another point of significance to the discussion of bloom sequence control, Section IV.

After the blue-green population had dropped in February, 1973, the waters of the second winter sustained a fairly high population of flagellates, and this mixed flagellate population persisted thru the spring of 1973.

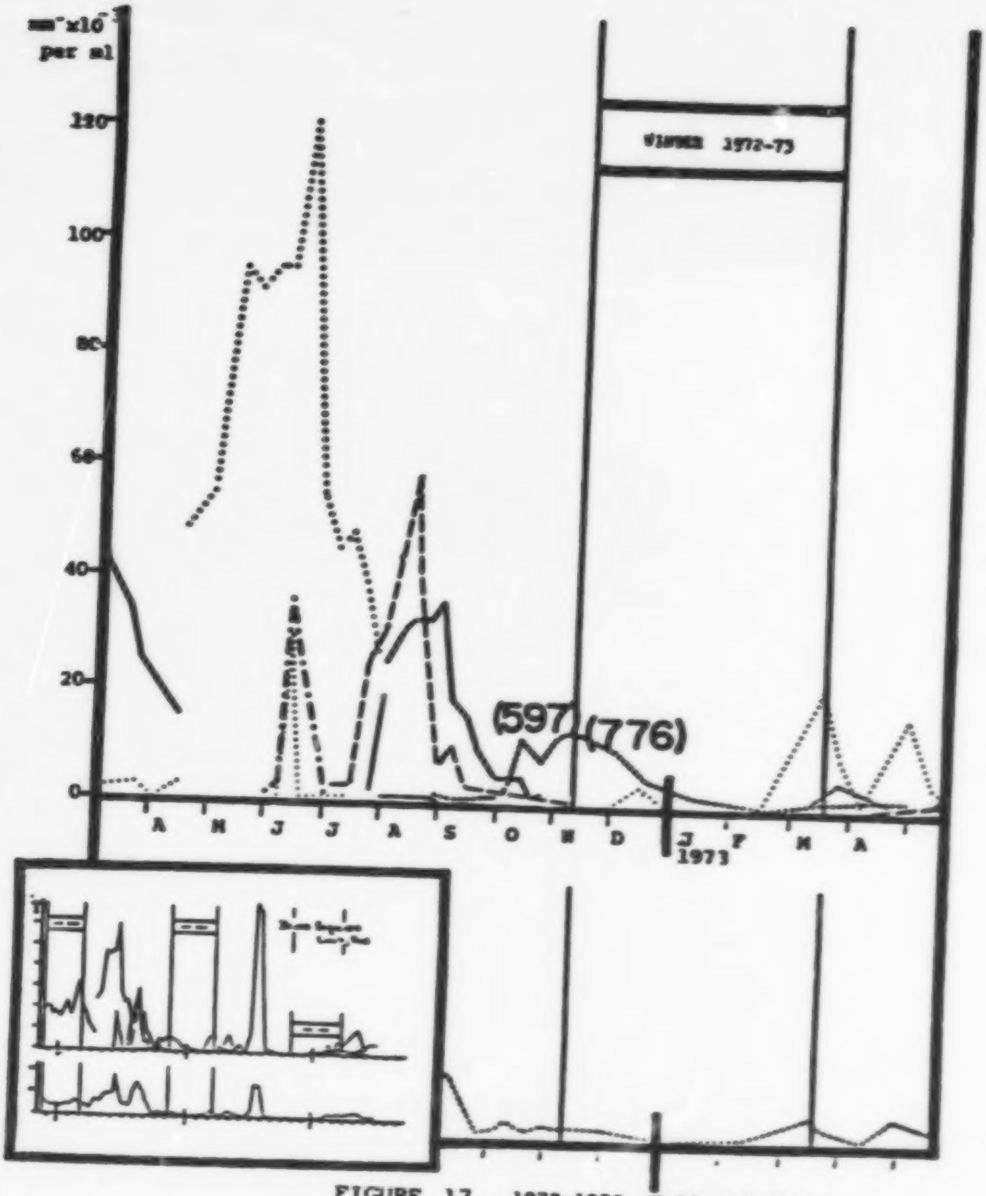


FIGURE 17. 1972-1973, Fall and Winter.
Insert for comparison.
597-Pseudanabaena galeata
776-Oscillatoria sp.

1973, Spring and Summer

The first diatom bloom to occur during this study was produced in the spring of 1973 (Figure 18). This bloom was dominated by *Asterionella formosa* (800), and persisted from early March to mid June, 1973. A sudden drop in the graph in early May (Figures 12 and, or, 18) reflects heavy rain and the washing out, by heavy runoff, of the *A. formosa* (800) population. The timing of the field trip at that point in the graph was such that *A. formosa* (800) gave the appearance only of waning. The occurrence of *Synechecoccus sp*. (91) at that time may have been partially responsible for the slow recovery of *A. formosa* (800) population since *Synechecoccus sp*. (91) filtrates consistently produced a negative effect on the growth of *A. formosa* (800) in filtrate studies.

The diatom bloom ended approximately three weeks later in mid June, 1973. This was at the same time as a drop in available silica. Data supporting the premise of silica limitation of the diatom populations in Linsley is included in Section VII, and is also discussed in Section VIII.

The onset of an exceptionally dense bloom of the very large *Anabaena sp*. (765), which had appeared in late summer, 1972, marked the end of desirable conditions in the 1973 growing season. In mid September, 1973, the waters of Linsley Pond were once again relatively free of phytoplankton.

1973-1974, Fall and Winter

Although *O. rubescens* (535) was present in low numbers from January thru March, 1974, neither of the *O. rubescens* strains (535, winter; 739, summer) from the first annual cycle (1971-1972) appeared in quantity during the 1973-1974 fall and winter; nor did either appear in quantity during the preceding (1973) growing season (Figure 19). *Pseudanabaena galeata* (597) and *Oscillatoria sp*. (776) of the second fall and winter cycle (1972-1973) were also present but only in low numbers in this third winter (1973-1974). The combined populations of (597) and (776) in the late winter and early spring are shown on the graph as a single double-peaked bloom. They could not be securely distinguished during counting procedures. Generally, however, when compared to the previous two winters (Figure 12), Linsley was relatively free of blue-green blooms in the third winter.

1974, Late Winter and Spring

The population of flagellates in this third winter was similar to, but less than, that of the second winter. The diatom occurrence, however, was quite distinct from all previously monitored seasons. In mid January diatoms began to appear in Linsley. By late February a bloom population was established (Figure 20). This bloom included *Asterionella formosa* (800), the dominant form in the less extensive 1973 spring diatom bloom, but was essentially a mixed diatom bloom. Most forms resembled *Synedra* and, or, *Fragilaria*. This mixed population, which persisted until early

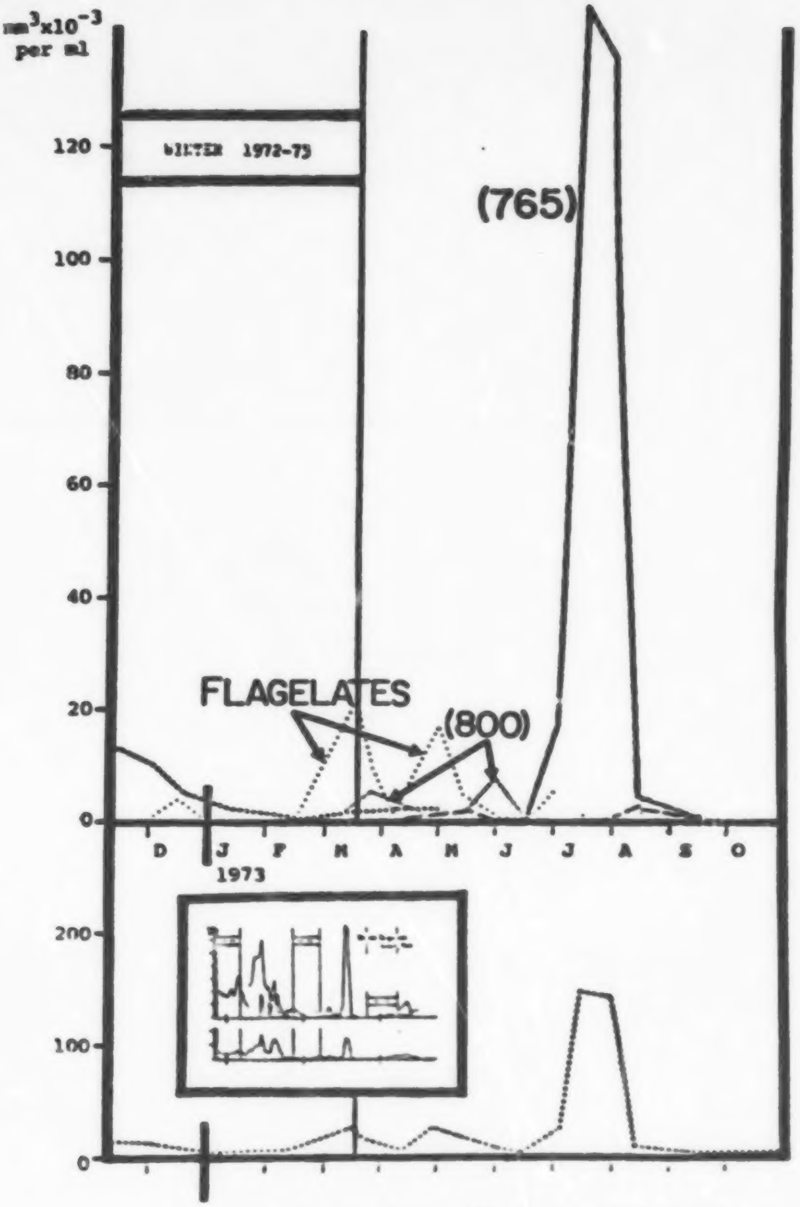


FIGURE 18. 1973, Spring and Summer.
Insert for comparison.

765-*Anabaena sp.*
800-*Asterionella formosa*

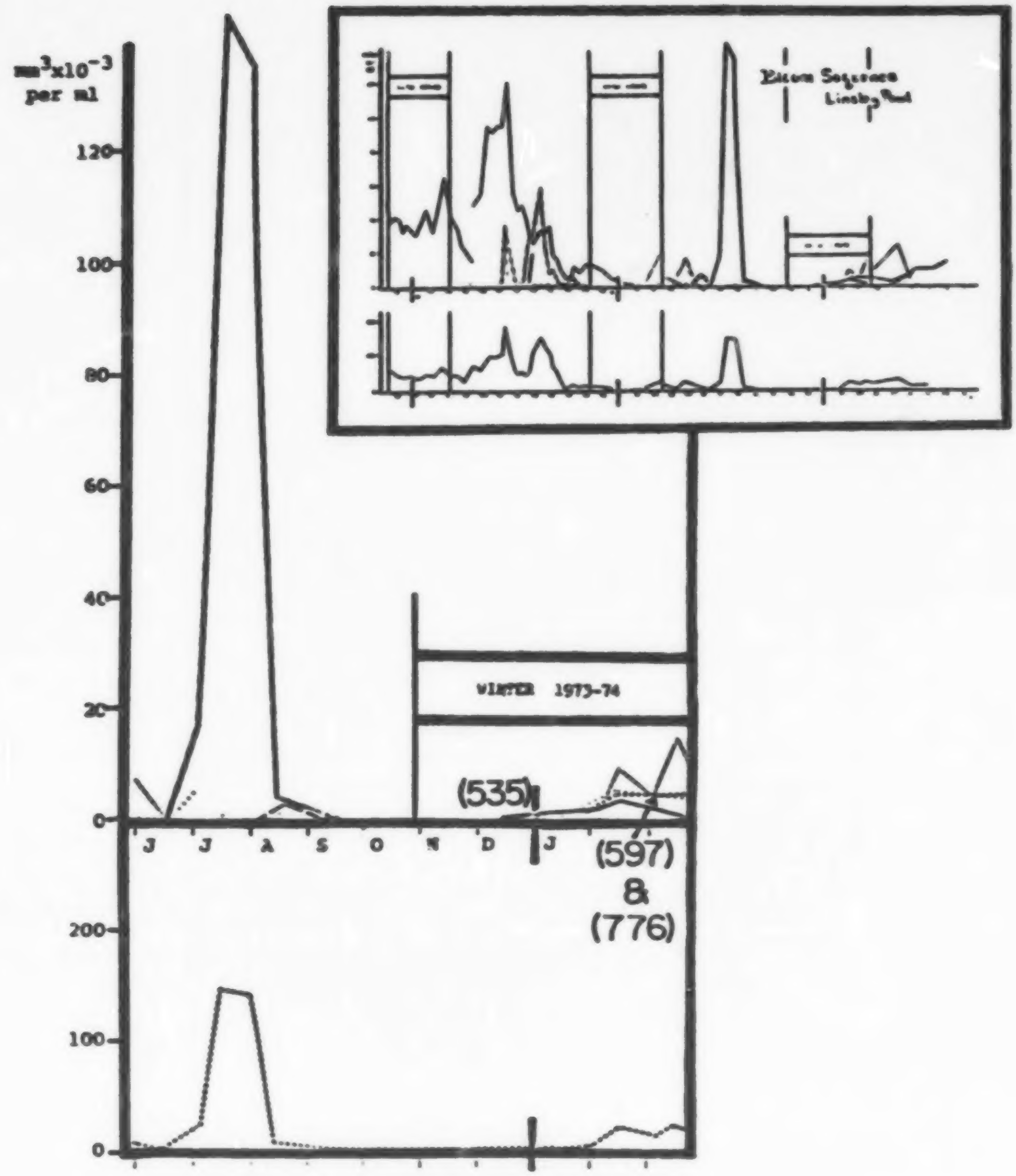


FIGURE 19. 1973-1974, Fall and Winter. Insert for comparison.

535-O. rubescens
597-Pseudanabaena galeata
776-Oscillatoria sp.

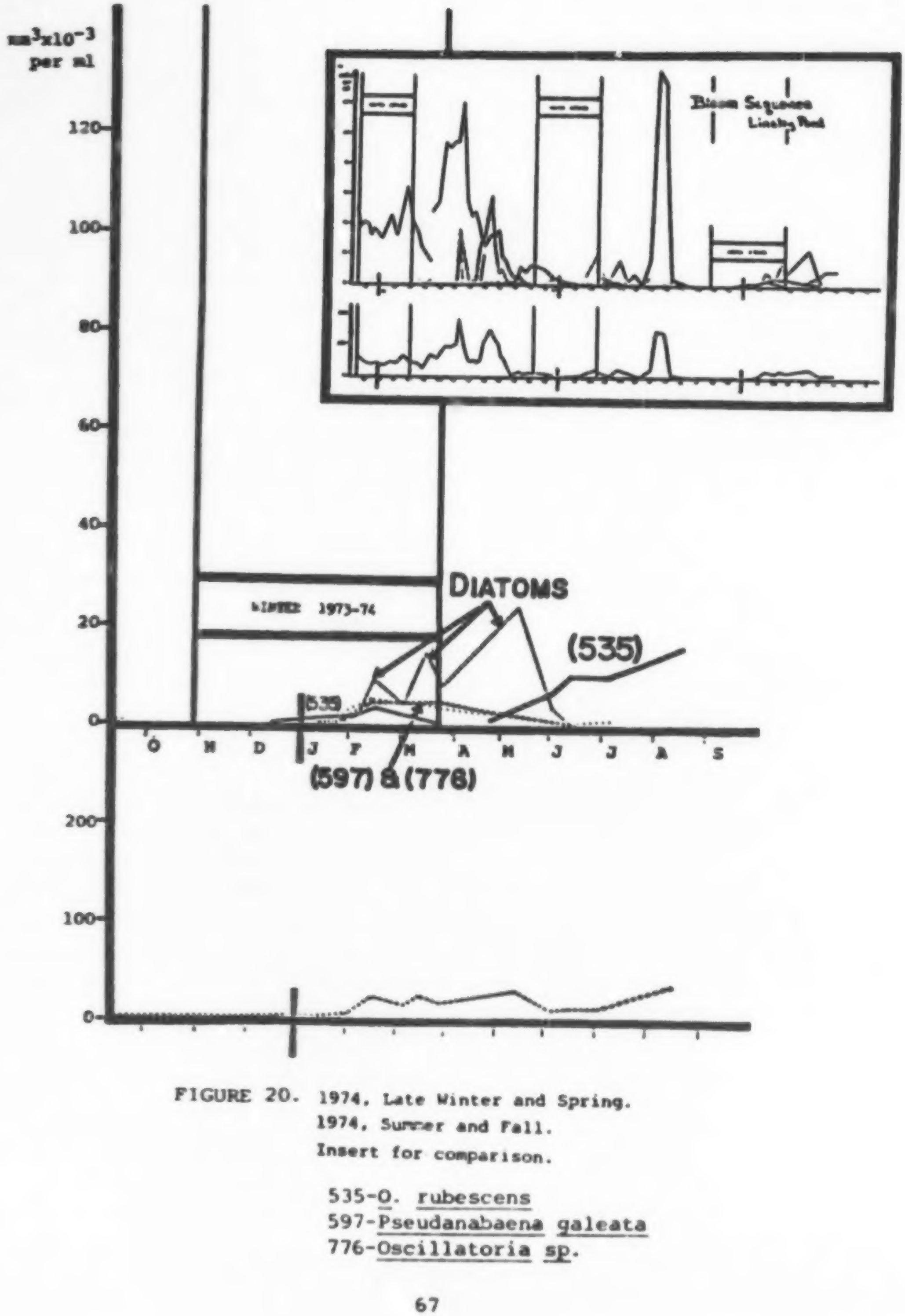


FIGURE 20. 1974, Late Winter and Spring.
1974, Summer and Fall.
Insert for comparison.

535-*O. rubescens*
597-*Pseudanabaena galeata*
776-*Oscillatoria sp.*

June, was consistently well distributed thru the water column.

As in the spring of 1973, the 1974 spring diatom bloom ended in early June when the available silica level was insufficient to sustain growth and reproduction in the large diatom population, see Section VII:V for further consideration of this point.

1974, Summer and Fall

In early June the mixed populations of flagellates and diatoms waned, and Oscillatoria rubescens (535) once again appeared in Linsley (Figure 20). This appearance of O. rubescens (535) was quantitatively less than the O. rubescens (739) bloom two years before the summer of 1972. It also differed in a qualitative way; i.e., it appeared only in the deeper waters, whereas the (739) bloom in 1972 maintained a depth of 0-3m. Whether, or not, the first (535) bloom (fall, 1971) was also distributed thru the water column is uncertain because samples taken during the very first field trip were only from shore points. The surface waters of Linsley during the latter part of the summer of 1974 appeared to be free of algal blooms. The deeper waters, however, maintained a fairly dense population of O. rubescens (535).

By the end of the summer in 1974 a high population of O. rubescens (535) had developed in the hypolimnion of Linsley. The presence of H_2S did not seem to hamper the development of these organisms in these deep and relatively dark (well below the 5% of ambient light—Secchi depth) waters of the pond. In the laboratory both of the O. rubescens strains, but especially (535), establish high populations in moderate or subdued light (10 to 20 foot candles).

Figure 21, below, locates the depth of the late summer-fall population of O. rubescens (535) and compares this distribution to the H_2S horizon. The population is graphed both by cell count and by O.D. The similarity of these curves above the H_2S horizon attests to the validity of O.D. measures as approximations of cell counts (especially since these values are within the least accurate part of the O.D. range, accuracy and repeatability improve at higher values). The consistent O.D. reading of 0.015 below the H_2S horizon reflects the even distribution of bacteria thru the hypolimnion. Waters appeared cloudy when test tube samples were examined visually. The O.D. readings above zero in the 0-3.5m depths reflect the presence of phytoplankters other than O. rubescens.

This winter, 1974-1975, the O. rubescens (535) bloom survived thru turnover and thru the winter months, but did not equal the density of the 1971-1972 winter (535) bloom. A diatom bloom dominated by Asterionella formosa (800) developed in the spring. Thus the moderate winter blue-green growth in the waters of both 1972-1973 and 1974-1975 allowed an A. formosa (800) dominated spring diatom bloom to develop suggesting a greater tolerance for blue-green allelopathic effects may exist in A. formosa than in the other diatoms of Linsley.

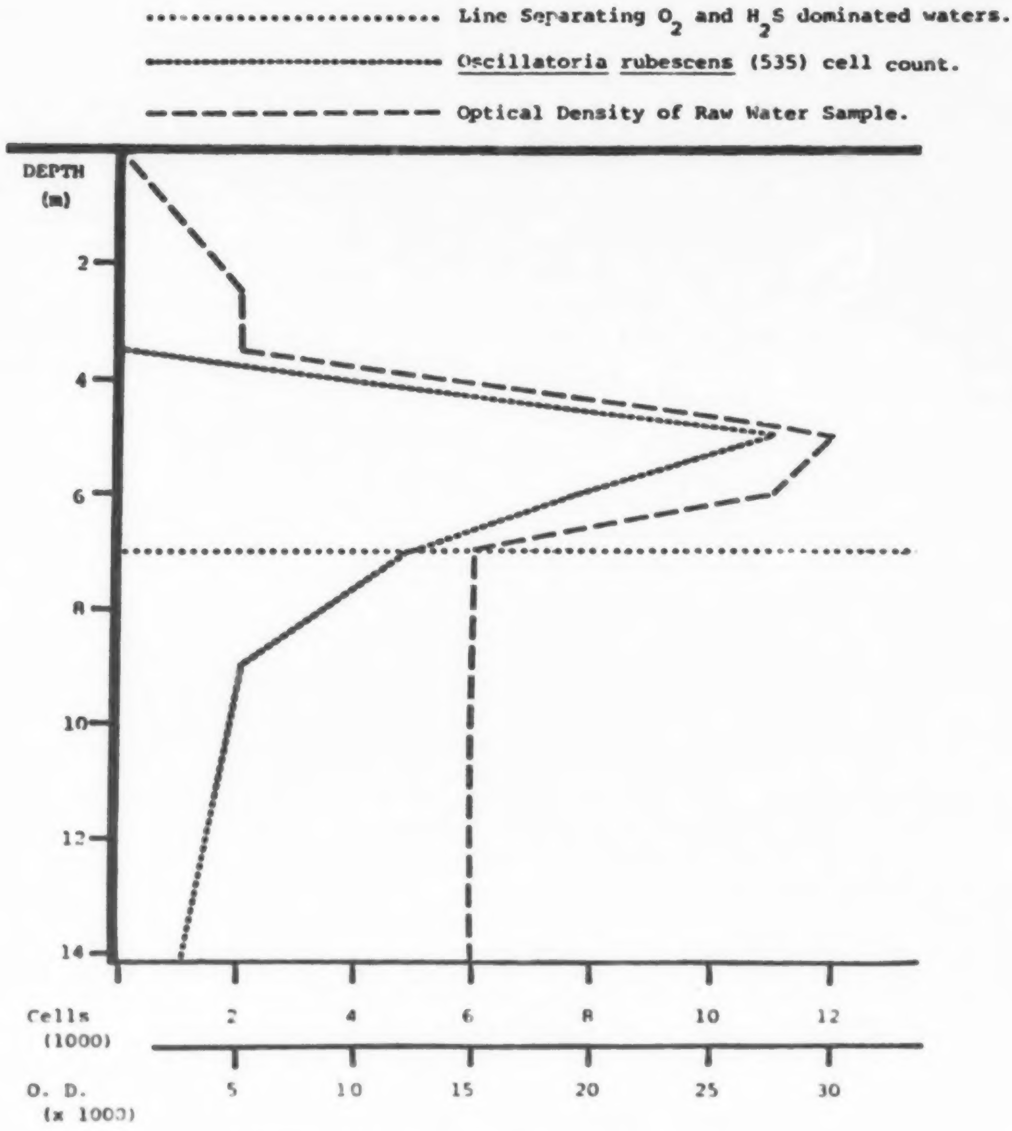


FIGURE 21. Oscillatoria rubescens. Depth of natural population compared to depth of H_2S boundary. (Cell Count and O. D.)

1971-1973, Zooplankton Populations

The zooplankton population of Linsley Pond is reported for the first two years of this study. The contrast between these two years prompted the focus of attention on ths zooplankton (Figure 22).

During the first year very few zooplankters were noted even in the large surface samples which were collected for bioassay. In contrast high populations were observed in all samples during the spring of the second year. With the onset of the *Anabaena sp.* (765) bloom in mid June, 1973, the zooplankton population once again dropped to such low levels that none, or only a very few, were noted even in the large bioassay samples.

This unexpected, high population of zooplankters was accompanied by the first (during the study period) signs of healthy fish in the pond. Along the shores of the lake not only a variety of minnows and catfish, but also a large population of blue-gills, who had carefully staked out claims to all available territories along the Yale landing, appeared. Although there were supposedly fish in the lake in the first summer, not a single living specimen was observed either along the shore or in the open water during any of the weekly field trips. There were, however, quite a few dead fish noted in the first year.

In general the fish population appeared far more numerous, and healthier, in the summer of 1973 than they had in the summer of 1972.

C = Cyclops Observed in Sample

B = Eubosmina Observed in Sample

D = Ceriodaphnia Observed in Sample

Each Rectangle above a Letter indicates Approximately 200/Liter

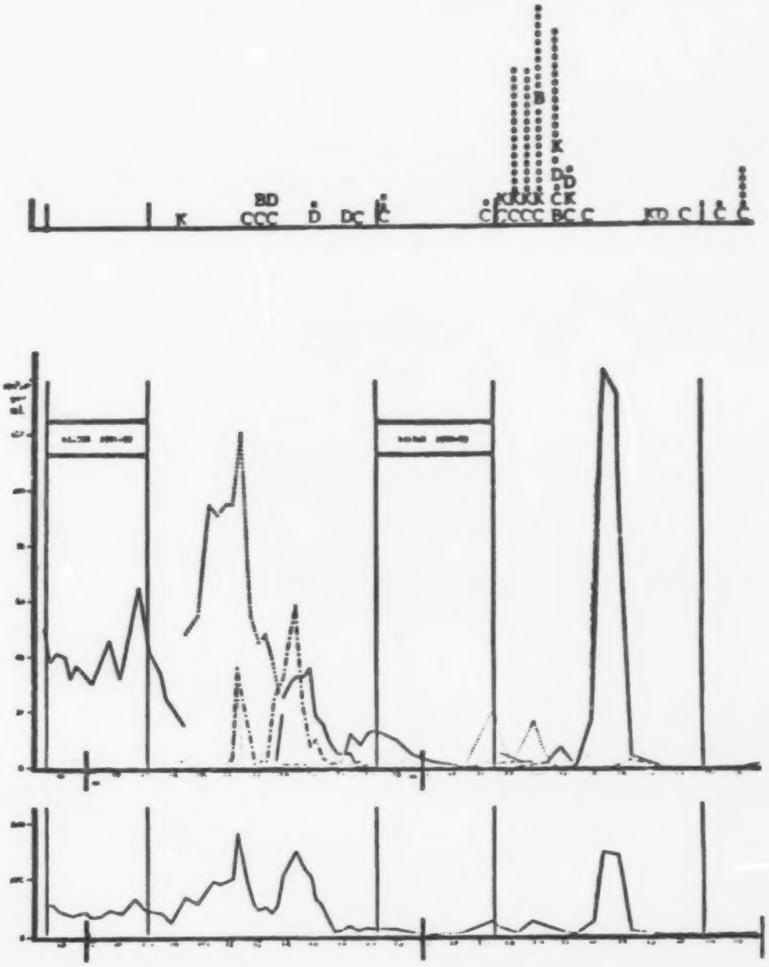


FIGURE 22. Zooplankton occurrence compared to blue-green algal occurrence 1971-1973.

SECTION VII

LABORATORY STUDIES

VII:I BIOASSAY

Culture Collection

Isolation—A variety of methods were employed to isolate phytoplankton from the pond. Larger forms were picked from pond water samples, washed, and placed in separate test tubes containing 10 ml portions of enriched pond water (enrichment was ES_I, described under Maintenance, below). Smaller forms were isolated by plating several drops of freshly collected pond water on Oxoid, Specially Purified, Ionagar #2, with various nutrient additions; or by inoculating test tubes containing 10 ml aliquots of various nutrient media with 0.1 ml aliquots of freshly collected water. Inocula were taken from 0, 2.5, 5, 9, and 13.5 meter pond water samples. The basic liquid to which agar and, or, nutrients were added was either distilled water, or pond water from 0, 2.5, 5, 9, or 13.5 meters. Agar was washed (Sands and Bennet, 1964) or unwashed. In some cases pH was controlled and intentionally set at one unit intervals from 6 thru 11. Nutrient additions included various combinations of vitamins and inorganic salts. Many of the combinations employed have been published as growth media, or as useful media additions. A listing of media employed may be found in Appendix B. The most universally successful growth medium is a 10 ml aliquot of charcoal treated pond water with 1.5% ES_{Isi} enrichment (see details of preparation under Maintenance, below).

The great variety of nutrient enrichments produced an equal variety of dominant organisms in these mixed cultures of pond organisms. Unialgal cultures were obtained from these primary cultures, or in some cases from secondary, or tertiary, cultures, by picking organisms (with a drawn glass micropipette) from promising colonies either on the agar plates or in the test tube cultures. Colonies were selected at 200x or 400x, but physical removal of selected organisms with the pipette was done under 100x or 200x using an inverted Leitz microscope.

Any culture labelled "clone" is the result of a single cell (or single organism in the case of multicellular forms) producing a new culture after having been isolated in a test tube containing 10 ml of enriched pond water.

Maintenance—Stock cultures are maintained at 18-23°C, under 10 200 foot candles of light, in a natural pond water medium designated "B medium". B medium is prepared with 50% pre-fall turnover pond water, preconditioned by one year (or more) of storage in darkness at approximately 5°C, and 50% post-fall turnover pond water similarly preconditioned. Pond water portions are charcoal treated (4 grams powdered, activated charcoal per liter, stirred constantly for one hour, passed thru a Millipore, H.A., 0.45μ filter), mixed, distributed into 10 ml test tube portions, and autoclaved for 20 minutes. ES_{Isi} at 1.5% (ES_I, Provasoli, 1968: with 150 ml% silica, as $Na_2SiO_3 \cdot 9H_2O$ added to the basic ES_I formula) is added asceptically just prior to inoculation of cultures.

Axenization—The elimination of bacterial contaminants from unialgal cultures was accomplished either by streaking on agar and picking clean colonies, or by ultra-violet treatment. Washing, either by repeated transfer of organisms of interest from sterile bath to sterile bath, or by allowing organisms of interest to rest upon filters which are profusely flushed with sterile medium, did not prove useful when dealing with blue-green algae (it was not necessary with eukaryotic algae), probably because the mucilaginous sheath permits a very close association between the alga and its accompanying bacterial community.

Treatment of bacterized cultures with ultra violet light proved an effective method of axenization. A 10 ml culture of the organisms of interest was poured into a 10 ml covered Petri dish, a 3 cm stirring bar was added, and the covered Petri dish was placed on a magnetic stirring table. This assemblage was then placed under a transfer hood equipped with a u.v. tube which was approximately 20 cm above the dish. The cover of the Petri dish was removed and the contents of the dish were exposed to the u.v. light for one minute intervals. After each such exposure a small sample of the culture was aseptically removed and inoculated into a test tube containing 10 ml of sterile B medium.

Exposure times ranged from 1 to 20 minutes. In the most successful attempts a minimum exposure of five minutes was necessary to eliminate bacterial growth. In several attempts bacteria survived as much as 20 minutes of u.v. exposure. Actually, the u.v. treatment often proved more lethal to the prokaryotic algae than to bacteria. The condition and age of cultures, and a degree of chance, determined the outcome of such efforts. The assumption of bacteria-free status was based on the methods described under Sterility Testing, below.

Organisms—At minimum the following families are represented in the Linsley Pond culture collection:

MYXOPHYTA (blue-greens)

Chroococcaceae, Oscillatoriaceae, Nostocaceae

EUGLENOPHYTA

Phacotaceae, Euglenaceae

CHRYSOPHYTA (diatoms)

Synuraceae, Coscinodiscaceae, Tabellariaceae, Diatomaceae,
Fragilariaceae, Naviculaceae, Surirellaceae

CHLOROPHYTA

Chlamydomonadaceae, Volvocaceae, Tetrasporaceae, Oocystaceae, Scenedesmaceae, Ulotricaceae, Trentepohliaceae, Cladophoraceae, Zygnemataceae, Desmidiaceae.

Due to the difficulties involved in, and the time consumed by, the proper taxonomic treatment of an algal organism, there are many cultures which have not been identified as to genus or species with certainty. Quite naturally those organisms which have been employed in assays were given priority and were more certainly placed taxonomically than were those which have not yet been employed experimentally. Unialgal cultures which represent unique organisms were routinely added to the culture collection. The intent is the inclusion of as many members as possible of the planktonic (a few of the cultured organisms are from littoral mats) community of this single pond so that they are available in culture for future experimentation.

For the same reason a _Eubosmina_ and a _Ceriodaphnia_ from Linsley are maintained in culture. These two, along with a _Keratella_ and a _Cyclops_, are the major zooplankters of the pond. Although _in vitro_ experiments can never be assumed to provide information which related directly to an _in situ_ event, the possibility that valid information may be obtained is increased when organisms from the same location are employed. This is particularly significant when strains, or "variants", of algae are involved since algal strains differ not only in nutritional and environmental requirements but also in the array and quantity of metabolites produced.

Dr. Stjepko Golubic and Dr. Francis Drouet provided guidance with the taxonomic identification of blue-green algae of significance to this study and Dr. Ruth Patrick provided similar aid with the diatoms of significance. Additional taxonomic information relative to the blue-green (Myxophyta) algae employed in this study may be found in Keating (1975, Appendix C).

Sterility Testing—The determination of bacteria-free status was done both visually via light microscopy (aided by dark field, phase contrast, and, or, oil immersion techniques), and biologically via inoculation of small aliquots of cultures to be tested into semi-solid test media consisting of 0.25% Oxoid Ionagar #2 in D.A. or S.T.P. media (see Appendix B for nutritional complement of media).

Filtrate Production

A series of blue-green algae have been employed as producers of filtrates. Those of Linsley origin have all produced bloom populations during the last three years. The two non-Linsley blue-greens employed were: 1) _Nostoc muscorum_ from the Indiana Collection via Dr. Luigi Provasoli's culture collection; and 2) _Nostic sp_. via Edward Bonneau, from the Connecticut

River cultures established and maintained by Peter Bonanomi.

Filtrate Producing Organisms:

Oscillatoria rubescens de Candolle (winter strain) (O. agardhii) (535)*
filamentous, single trichomes, planktonic, non-heterocystous, green-brown, blooms in fall thru winter, bacterized, very obvious phototrophic response.

Oscillatoria rubescens de Candolle (summer strain) (O. agardhii) (739, 746)
filamentous, single trichomes, planktonic, non-heterocystous, orange-green-brown, blooms in spring thru midsummer, bacterized, no obvious phototrophic response.

Pseudanabaena galeata Bocher (597)
filamentous, single trichomes, planktonic, non-heterocystous, vivid blue-green, blooms in early fall thru early winter, axenic (this may be the organism originally designated as the type organism for Oscillatoria rileyi)

Oscillatoria sp. (776)
filamentous, single trichomes, planktonic, non-heterocystous, green-brown, blooms in mid winter, axenic.

Anabaena sp. (538)
filamentous, single trichomes, planktonic, with heterocysts, green, blooms in late spring, axenic.

Anabaena sp. (762) or Aphanizomenon elenkinii Kisel (762)
filamentous, forms flakes, planktonic, with heterocysts, green, blooms in late summer (minimal bloom in 1972), bacterized.

Synechecoccus sp. (91)
coccoid, single cells, planktonic, green-blue-green, blooms in spring, axenic.

Nostoc muscorum
non-Linsley, filamentous, single trichomes, heterocystous, deep-green, Indiana collection, axenic.

Nostoc sp.
non-Linsley, filamentous, colonial, probably non-planktonic, heterocystous, green, collected from Connecticut River 1969, from the collection of Peter Bonanomi, University of Connecticut, forms globular colonies, axenic.

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*Numbers—Linsley Pond culture collection designation.

Thawing of Stored Pond Water

Large samples of pond water were filter-sterilized and stored at -20°C immediately after collection. These samples were later thawed and assayed for biological activity. Individual 2.5 liter polyethylene containers were placed in supportive dishes and allowed to thaw overnight at room temperature (16 hours at 22°C). Prior to processing the sample into F vs A assays, a sterility test was taken to assure that the sample had remained sterile during storage.

Assay System

Basis—preliminary work (involving isolation of various algal species) indicated that population maxima in unialgal cultures established in pond water differ, and that this difference depends on the treatment of the pond water prior to inoculation. Pond water was either 1) filter-sterilized; 2) filter-sterilized and autoclaved; 3) charcoal-treated and filter-sterilized, or 4) charcoal-treated, filter-sterilized, and autoclaved. Further study indicated that charcoal treatment (described above under *Culture Collection: Maintenance*, and originally employed because it was found to remove most traces of certain organic compounds from pond water) added materials to the pond water. However, there remained a consistent, obvious, difference between population maxima in filter-sterilized, and filter-sterilized and autoclaved, water samples. This suggested the presence in the pond water of heat-labile compounds worthy of scrutiny, and this possibility initiated the pattern for assay followed throughout this study.

The Assay— the assay is based on a comparison of the growth of selected organisms in test tubes containing 10 ml portions of pond water. The pond water to be tested is either filter-sterilized, or filter-sterilized and autoclaved (*these two treatments are usually simply referred to as F and A*) prior to inoculation with assay organisms. 1.5% ES_I enrichment (no silica addition) is added just prior to inoculation of cultures.

This assay is also employed when the biological activity of producer filtrates is studied. The term "producer filtrate" (or "filtrate") refers to that water in which a culture of an alga has been grown and from which the "producer" algae have been removed by passage thru a 0.45μ (H.A.) Millipore filter. These large producer cultures are established in B medium, described above under *Maintenance*.

In order to allow the pH of autoclaved portions to return to the original F level, a minimum of one week was allowed to pass between autoclaving and inoculation. This return was consistent and no pH distinction between F and A (filtered and autoclaved) tests greater than 0.1 pH units was noted. During this equilibration period the tests were stored in test tubes at 0-2°C in darkness.

Assay Organisms—a taxonomic variety of diatoms, blue-green algae, green algae, and flagellated forms were used as assay organisms. A listing of assay organisms is included in Appendix C. The selection of assay organisms depended on the specific information sought in a given set of experiments.

Growth maxima were determined by O.D. readings. A densitometer which accommodates test tubes rather than cuvetts, and which was built by Dr. Fred Kavanaugh for Haskins Laboratory, was employed for all readings of growth maxima.* The capacity of this meter to accept test tubes permitted O.D. readings to be taken without decanting (and contaminating) the growing cultures and, thereby, permitted constant surveillance of culture growth over extended periods of time (usually several weeks). Immediately before O.D. readings were taken the test tube containing the culture to be measured was thoroughly shaken on a Vortex Genii.

Light and Temperature—cultures and experimental tests were grown under 24 hour light provided by cool white fluorescent bulbs. During tests light intensity varied between 400 and 600 foot candles. This light variation was dependent on both the age of an individual light tube and on the placement of a specific test tube either directly over (illumination was from below during tests), or one to five cm to either side of, a light tube. Since it was impractical to eliminate these light variations, each set of test tubes which was to be used as the basis of a single comparison and interpretation of F vs A growth was carefully, and consistently, placed in the incubator to minimize or eliminate this variation; i.e., each such set of tubes was always placed parallel to, and equidistant from, the light tube. If light variation was due to the aging of a light tube, all tests in a set experienced a similar variation.

Glassware—for experiments 10 ml portions of media were employed in 20 x 125 Pyrex test tubes with linerless screw caps. All test tubes were scrubbed clean in a solution of 7X, rinsed in tap water, and washed in an automatic dishwasher (using recommended concentrations of 7X for automatic washing) with a 30-minute wash, 30-minute rinse, and 30-second distilled water rinse. Tubes were then heated for a three-hour cleaning-cycle in a General Electric oven equipped with P7 (electric oven cleaning). This baking represents over 2.5 hours at a temperature between 900-1000°F. The intent of such heat treatment was to assure disruption of the carbon backbone of all organic compounds which might be present in test tubes. After baking, tubes were rerinsed in the automatic dishwasher in distilled water for 30 seconds (to remove any film of ash remaining after baking, no visible film was noted) and were dried in an oven.

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*Additional information relative to this O.D. meter may be found in Appendix D.

Filtrate cultures were grown in 1000 ml portions of B medium in 2500 ml Corning #4422 Pyrex extra wide, conical, culture flasks. Culture flasks (and all other incidental glassware used for preparing and distributing media) were scrubbed in a 7X solution, rinsed in tap water, rerinsed in distilled water, and allowed to air dry. Culture flasks were purchased new and were employed solely for filtrate production.

VII:II BIOLOGICAL ACTIVITY IN FILTRATES OF BLOOM DOMINANT ORGANISMS AND IN NATURAL POND WATERS

Heat-Labile Biological Activity in Filtrates of Bloom Dominant Blue-Green Algae

Filtrates of blue-green algae were tested against a variety of assay organisms. The results are summarized in Table 5. The blue-green filtrates produced a complicated array of positive, negative, and neutral effects on the assay organisms employed. A generalized negative effect of blue-green filtrates on diatoms was noted quite early in the study, and this phenomenon was singled out for study in depth. This study is summarized below in Sections VII:III and VII:IV.

Because similar results obtained from separate filtrates are more valid than similar results obtained from replicate tests of the same filtrate, at least two separate filtrate cultures (from two to twenty-two) were established and harvested for each of the bloom dominants. Also, replicate tests were employed for each of the separate filtrate studies. With the exception of one species, Anabaena sp. (765), which was difficult to maintain in culture, and which was not successfully grown in a culture suitable for filtrate production, each of the blue-green organisms which were observed to be bloom dominants during the study period (1971-1974) was employed as a filtrate producer. In any study of a single filtrate four separate tests (2F and 2A) of any organism employed for assay were run in parallel. Due to time and space limitations only one filtrate culture (1000 ml) was produced for Anabaena sp. (762) and for each of the non-Linsley blue-greens. In these cases 4F and 4A tubes were included for each organism tested.

The number of filtrates tested for each producer organism is listed in Table 5 in the lower left hand corner of the PRODUCER box (first column on left). The name, Linsley culture identification number, and condition (axenic or bacterized) of each filtrate at harvest are also included in this PRODUCER box. Because it was not always possible to assay every sensitive organism when a given filtrate was being studied, most of the assay categories (four columns on right—Table 5, under the heading ASSAY ORGANISMS) involved less than the total number of filtrates produced by the particular producer organism involved. This is especially true of Anabaena sp. (538) which produced 22 filtrates. In order to convey the number of filtrates actually used in any given assay category, the number of filtrates used in the determination of effects for each of the ASSAY

TABLE 5. SYMBOLS

Neg., Pos., Neu. = Filtrate had a negative, positive, neutral effect on assay organisms.

(Neg.), (Pos.), (Neu.) = Filtrate had a very weak or uncertain negative, positive, neutral effect on assay organism.

Numbers

- Lower left hand corner of PRODUCER name box = number of separate filtrates of the organism harvested.

- Upper right hand corner of effects box:
  - top number = number of filtrates used for
  - second number = number of different organisms used for
    - Organisms are listed by name for each set of assays,

- Immediately following symbol for effect on assay organism (i.e., pos., neg., neu.) = the number of assay organisms giving that result.

TABLE 5. Summary: Heat-Labile Biological Effects of Blue-Green Filtrates.

PRODUCER	ASSAY ORGANISMS			
(Axenic) (Bacterized)	DIATOMS	BLUE-GREENS	GREEN ALGAE	FLAGELLATES
Oscillatoria rubescens (535) Bact. 4	Neg. 5 $\genfrac{}{}{0pt}{}{4}{8}$ Neu. 3	$\genfrac{}{}{0pt}{}{3}{8}$ Pos. 5 (Neu.) 3	(Neg.) 1 $\genfrac{}{}{0pt}{}{2}{3}$ Pos. 2	(Neg) 1 $\genfrac{}{}{0pt}{}{2}{10}$ Pos. 6 Neu. 3
Oscillatoria rubescens (739) Bact. 2	Neg. 6 $\genfrac{}{}{0pt}{}{2}{6}$	Neg. 3 $\genfrac{}{}{0pt}{}{2}{8}$ Pos. 3 Neu. 2	(Neg.) 1 $\genfrac{}{}{0pt}{}{1}{3}$ (Pos.) 2	(Neg) 1 $\genfrac{}{}{0pt}{}{1}{10}$ Pos. 3 Neu. 6
Anabaena sp. (538) 22 Ax.	Neg. 29 $\genfrac{}{}{0pt}{}{22}{29}$	Neg. 3 $\genfrac{}{}{0pt}{}{5}{8}$ Pos. 3 Neu. 2	Neg. 2 $\genfrac{}{}{0pt}{}{2}{13}$ Pos. 3 Neu. 8	omit
Pseud- anabaena galeata (597) Ax. 2	Neg. 7 $\genfrac{}{}{0pt}{}{2}{8}$ (Neu.) 1	Neg. 4 $\genfrac{}{}{0pt}{}{2}{8}$ (Pos.) 1 Neu. 3	(Neg) 2 $\genfrac{}{}{0pt}{}{2}{9}$ Pos. 7	Neg. 2 $\genfrac{}{}{0pt}{}{1}{4}$ Neu. 2
Oscillatoria sp. (776) Ax. 2	(Neg.) 5 $\genfrac{}{}{0pt}{}{1}{6}$ (Neu.) 1	Neg. 6 $\genfrac{}{}{0pt}{}{1}{8}$ (Neu.) 2	Neg. 5 $\genfrac{}{}{0pt}{}{2}{6}$ (Neu.) 1	Neg. 2 $\genfrac{}{}{0pt}{}{1}{6}$ (Pos.) 1 (Neu.) 3
Syneche- coccus sp. (91) Ax. 2	Neg. 9 $\genfrac{}{}{0pt}{}{2}{9}$	Neg. 4 $\genfrac{}{}{0pt}{}{2}{8}$ Pos. 4	Neg. 2 $\genfrac{}{}{0pt}{}{2}{7}$ Pos. 3 Neu. 2	Neg. 2 $\genfrac{}{}{0pt}{}{1}{5}$ Pos. 3
Aphani- zomenon flos-aquae (766) Bact. 2	Neg. 4 $\genfrac{}{}{0pt}{}{2}{7}$ Neu. 3	Neg. 2 $\genfrac{}{}{0pt}{}{1}{8}$ Pos. 3 Neu. 3	$\genfrac{}{}{0pt}{}{1}{6}$ Pos. 3 (Neu.) 3	$\genfrac{}{}{0pt}{}{1}{5}$ (Pos.) 1 Neu. 4
Anabaena sp. (762)	Neg. 2 $\genfrac{}{}{0pt}{}{1}{3}$ (Pos.) 1	Neg. 5 $\genfrac{}{}{0pt}{}{1}{8}$ Pos. 3	Omit	Omit
Nostoc muscorum (Indiana) Ax. 1	Neg. 4 $\genfrac{}{}{0pt}{}{1}{8}$ Neu. 4	(Neg.) 1 $\genfrac{}{}{0pt}{}{1}{8}$ Pos. 4 Neu. 3	(Neg.) 1 $\genfrac{}{}{0pt}{}{1}{3}$ (Neu.) 2	(Neg.) 1 $\genfrac{}{}{0pt}{}{1}{3}$ (Neu.) 2
Nostoc sp. (Bonanomi) 1 Ax.	Neg. 3 $\genfrac{}{}{0pt}{}{1}{8}$ Neu. 5	omit	omit	omit

ORGANISMS categories (diatoms, blue-greens, green algae, flagellates) is included as the top number of the right in each effects box (under ASSAY ORGANISMS) in Table 5. The second number on the right in this same box is the number of different assay organisms used for each category. (See Appendix C for a listing of organisms tested, and specific effects on each organism). The number following each of the symbols for negative, positive, or neutral (neg., pos., neu.) is the number of organisms responding in that manner to the specific filtrate.

Table 6, Effects of Blue-Green Algal Filtrates on Blue-Green Algae, is provided to permit a more detailed analysis of the interactions among the bloom dominants. The data for one of the non-Linsley blue-greens is included for comparison.

TABLE 6. SYMBOLS

-	The effect of producer filtrate on assay organism was negative.
+	The effect of producer filtrate on assay organism was positive.
0	The effect of producer filtrate on assay organism was neutral.
(-) (+) (0)	The effect was weak or uncertain.

TABLE 6. Effects of Blue-Green Algal Filtrates on Blue-Green Algae.

PRODUCER / ASSAY ORGANISM	Oscillatoria rubescens (535) winter	Oscillatoria rubescens (739) summer	Anabaena sp. (538)	Pseudanabaena galeata (597)	Oscillatoria sp. (776)	Synechecoccus sp. (91)	Aphanizomenon flos-aquae (766)	Anabaena sp. (765)
Oscillatoria rubescens (535) (535)	(0)	(0)	+	+	+	0	+	+
Oscillatoria rubescens (739) (739)	0	-	+	+	0	(-)	+	-
Anabaena sp. (538) (538)	+	-	0	0	-	+	+	-
Pseudanabaena galeata (597) (597)	0	0	-	(-)	(0)	+	-	-
Oscillatoria sp. (776) (776)	-	-	(-)	-	0	-	-	(0)
Synechecoccus sp. (91) (91)	(-)	-	-	-	+	+	+	+
Aphanisomenon flos-aquae (766) (766)	+	+	+	0	+	0	-	0
Nostoc muscorum (non-Linsley) (non-L)	+	0	+	+	+	-	0	(0)
Anabaena sp. (762) (762)	-	-	+	-	-	+	+	-

Heat-Labile Biological Activity of Freshly-Collected and Freezer-Stored Linsley Pond Waters

Freshly-collected and freezer-stored pond waters were assayed for biological activity. Results of these assays are summarized in Table 7. The number of assay organisms used in each of the assay categories (diatoms, blue-greens, green algae, flagellates) is included in each of the effects boxes (beneath the heading ASSAY ORGANISMS). The number following each of the symbols for negative, positive, or neutral is the number of organisms responding in that manner to the specific pond water sample. (See Appendix C for a listing of assay organisms used for both filtrate and pond water tests.)

Two assays were conducted using pond water collected on March 18, 1973. One of these assays was done with freshly-collected pond water and one with freezer-stored (and thawed) pond water. The intent of this duplication was to determine whether or not the freezing of pond water samples would significantly alter the heat-labile biological activity of the pond water (as demonstrated by diatom assays). In only one case, *Asterionella formosa* (800), was a different assay result indicated (Table 8). When freshly-collected pond water was assayed, *A. formosa* (800) grew slightly better in F than in A pond water samples; however, when freezer-stored pond water was assayed, *A. formosa* (800) grew considerably better in A than in F samples. This suggests a significant change in the heat-labile inhibitory capacity of this March 18, 1973 pond water during the freeze-store-thaw procedure. But closer examination of these results indicates that a different interpretation may be necessary because *A. formosa* (800) achieved similar growth maxima in the F assays of both portions (fresh and frozen) of the March 18, 1973 Linsley Pond water. There was, however, a significant difference in the growth maxima in the two A assays. Autoclaved samples of the freshly-collected pond water supported growth maxima similar to those of both types of filtered-only samples, however, autoclaved samples of the freezer-stored pond water supported more than twice that level.

Five other diatoms and one blue-green were also used in assays of these two portions of the March 18 water. All five of these diatoms produced a negative result in both freshly-collected and freezer-stored samples. One other of these five diatoms exhibited a change in autoclaved samples which was similar to the change in autoclaved samples of *A. formosa*; *i.e.*, in each case the autoclaved sample of freezer-stored pond water supported higher growth than did the autoclaved sample of freshly-collected water. This result suggests that during the freeze-store-thaw procedure a significant change occurred in some unexplored heat-stable inhibitor. That is, the heat-stable inhibitor expresses inhibition in freshly-collected samples, but loses some, or all, of its inhibitory potential during the freeze-store-thaw procedure. This interpretation requires the assumption that the heat-stable inhibitor is masked by the presence of the more potent heat-labile inhibitor in F assays of freshly-collected water. Other interpretations are possible.

TABLE 7. SYMBOLS

Neg., Pos., Neu. = Pond water had a negative, positive, neutral effect on assay organisms.

(Neg.), (Pos.), (Neu.) = Pond water had a very weak or uncertain (or only one organism was so effected) effect on assay organism.

Numbers in upper right hand corner of effects (assay organism) box = number of organisms tested.

Numbers immediately following symbol for effect on assay organism (i.e., pos., neg., neu.) = the number of assay organisms giving that result.

TABLE 7. Summary: Heat-Labile Biological Effects of Freshly-Collected, and Freezer-Stored Pond Waters.

DATE OF COLLECTION (fresh) (thaw)	ASSAY ORGANISMS			
	DIATOMS	BLUE-GREENS	GREEN ALGAE	FLAGELLATES
8/1/72 8/8/72 (fr)	1 (Neg.) 1	2 (Neg.) 1 (Pos.) 1	6 Neg. 4 Neu. 2	1 (Neg.) 1
9/5/72 (fr)	omit	4 Neg. 4	omit	omit
9/19/72 (th)	7 Neg. 7	8 Neg. 3 Pos. 2 Neu. 3	9 Neg. 3 Pos. 3 Neu. 3	7 Neg. 2 Pos. 3 Neu. 2
11/17/72 (th)	9 Neg. 8 (Neu.) 1	8 Neg. 4 (Pos.) 1 Neu. 3	10 Neg. 4 (Pos.) 1 Neu. 5	8 Neg. 3 Neu. 5
1/7/73 (fr)	3 Neg. 3	9 Neg. 3 Pos. 6	6 (Neg.) 1 Pos. 3 Neu. 2	1 (Pos.) 1
3/18/73 (fr)	18 Neg. 17 (Pos.) 1	1 (Pos.) 1	omit	omit
3/18/73 (th)	9 Neg. 9	8 Neg. 7 (Pos.) 1	9 Neg. 5 (Pos.) 1 Neu. 3	8 Neg. 4 (Pos.) 1 Neu. 3
5/13/73 (fr)	8 Neg. 8	omit	omit	omit
6/2/73 (fr)	9 Neg. 9	7 Neg. 2 Pos. 3 Neu. 2	2 (Pos.) 1 (Neu.) 1	omit

TABLE 7. (continued)

DATE OF COLLECTION (fresh) (thaw)	ASSAY ORGANISMS			
	DIATOMS	BLUE-GREENS	GREEN ALGAE	FLAGELLATES
7/17/73 (th)	7 Neg. 7	8 Neg. 3 Pos. 4 (Neu.) 1	6 (Neg.) 1 Neu. 5	9 Neg. 4 (Pos.)1 Neu. 4
8/13/73 (fr)	7 Neg. 7	5 Neg. 4 Neu. 1	2 (Neg.) 1 (Neu.) 1	omit
2/14/74 (fr)	8 Neg. 8	8 Neg. 6 (Pos.) 1 (Neu.) 1	8 Neg. 2 Neu. 6	4 Neg. 2 Neu. 2
6/13/74 (fr)	13 Neg.13	8 Neg. 5 (Pos.) 2 (Neu.) 1	9 Neg. 6 Pos. 2 (Neu.) 1	10 Neg. 8 (Pos.)1 (Neu.)1

TABLE 8. Maximum Growth as Expressed by O. D. for the Organisms Employed in F vs A Assays of Both the Freshly-Collected and the Freezer-Stored Portions of the Linsley Pond Water Sample Collected on March 18, 1973.

ORGANISM	FRESHLY-COLLECTED			FREEZER-STORED		
	F	A	Result	F	A	Result
Asterionella formosa (800)	65	55	(+)	75	145	(-)
Surirella sp. (352)	120	240	(-)	100	200	(-)
Synedra sp. (299)	60	110	(-)	65	140	(-)
Fragilaria sp. (99)	60	95	(-)	55	75	(-)
Cyclotella sp. (211)	80	110	(-)	135	270	(-)
Tabellaria sp. (764)	45	155	(-)	50	100	(-)
Synechecoccus sp. (91)	380	220	(+)	360	230	(+)

This is only one of the many indications of inhibitory and, or, stimulatory metabolite effects which are not heat-labile, and therefore, which have not been considered in this study. These numerous additional metabolite effects offer many challenging problems for future study.

VII:III PRELIMINARY CHARACTERIZATION OF THE HEAT-LABILE SUBSTANCE RESPONSIBLE FOR INHIBITION OF DIATOM GROWTH: METHODS

Methods described below were employed for individual experiments in conjunction with the activity bioassay described above. These experiments were designed to provide additional information relative to the inhibition of diatom growth potential by a metabolite of blue-green algae. Anabaena sp. (538) was employed as the producer organism due to its consistent growth characteristics and to its reliable production of filtrates.

Although a variety of diatoms were used as assay organisms Nitschia frustulum v. indica (Skvortzow) (224) was employed as the preliminary assay organism because of its relatively small size (good dispersal for optical density readings), consistently high population densities, and freen single cell, growth habit (in contrast to cells in a gelatinous matrix with, or without, attachment to glass vessel walls).

Heat Lability

In order to further examine the characteristics of the heat-labile diatom inhibitory capacity apparent in both Linsley Pond waters and producer filtrates a series of tests were pre-heated to 60°, 90°, and 121°C (autoclave). Tests were heated to designated temperatures and maintained at such for 20 minutes. The autoclaved tests were also subjected to the usual 15 lbs. pressure of the autoclave. Controls were non-heated, filter-sterilized water samples from the same source as the test cultures.

To provide additional insight into the effects of the temperature variations a dilution series was established for each of the target temperatures. As with other dilution series, tested test samples were mixed to designated dilution percentages with autoclaved portions of the same water as that of the test samples. Dilutions were 100%F, 66%F, 33%F, 0%F (0%F representing 100%A).

Volatility

To determine whether the heat-labile inhibition of diatom growth by filtrates of blue-green algae is the result of a volatile substance, N_2 at 20 psi was bubbled—via a Supreme "fine pore" aquarium air stone—thru a sample of filtrate for a period of 20 minutes. This sample was then prepared as a set of F vs A assays. For comparison and control a sample of the same filtrate (with no N_2 bubbling) was prepared as a set of the usual F vs A tests.

Dialysis

To determine whether the heat-labile inhibition of diatom growth by the filtrates of blue-green cultures is the result of a dialyzable molecule, a 1000 ml filtrate was dialyzed against sucrose; i.e., the 1000 ml filtrate was confined within the dialysis tubing and the tubing was buried under 5 lbs. of ordinary granulated Jack Frost refined sugar.

Approximately 100 ml of liquid remained inside the tubing after 24 hours. This was considered the "non-dialyzable" portion. The "dialyzable" portion of this filtrate was considered to be contained in that deep yellow-brown area forming a halo in the bed of wet sugar surround the tubing. This grainy yellow-brown portion of sugar was scooped up and added to a graduated cylinder. Its volume was then increased to approximately 100 ml by the addition of distilled water. Sugar granules were totally dissolved. This 100 ml dialyzable portion and 100 ml non-dialyzable portion were then separately filter sterilized by passage thru 0.45µ (H.A.) Millipore filters into sterile containers.

The amount of sugar in neither of these portions could be accurately determined with the equipment available; therefore, a comparison control was employed by the use of dilution series with the dialyzable and non-dialyzable portions serving as additions. An identical set of dilution series tubes was autoclaved and served as additional comparison and control. Dilutions were by additions of 0, 0.1, 0.5, 1.0, 2.5, and 5.0 ml of the dialyzable (or non-dialyzable) material to 10 ml aliquots of B medium. The final, most concentrated, test was set at 50% dilution by combining 5 ml of test solution with 5 ml of B medium.

There were thus four dilution series established:

-non-dialyzable F	-dialyzable F
-non-dialyzable A	-dialyzable A

Ultrafiltration

To determine the size of molecules involved in the inhibition of diatom growth by blue-green filtrates a series of ultra-filters were used to separate by size the metabolites contained in such filtrates.

Amicon ultrafiltrates were employed in an Amicon Model 52 stirred cell ultrafiltration unit. Filters and pore sizes (based on retention of protein molecules) were as follows:

	Particles Excluded	
	Size	MW
PM10	20 A°	10,000
UM2	10 A°	1,000
UMO5	-	500

Dilution series were established to determine the effects of each molecular size group. Dilutions were of F samples with A; 100%F, 66%F, 33%F, and 0%F (0%F representing a test with 100%A). The "A" used for dilution series was simply a portion of autoclaved filtrate; i.e., it was not autoclaved filtrate which had been passed thru the ultrafilters. To provide additional comparison and control a portion of the same filtrate was processed thru the usual F vs A assay.

Ether Extraction of Lipids

To determine if the substance responsible for the heat-labile inhibition of diatom growth by blue-green filtrates could be extracted by ether, a 300 ml portion of filtrate was extracted with 600 ml of Mallinckrodt Ether for Fatty Acid Extraction by shaking in a 4 liter separatory funnel for 45 minutes. The sample was then allowed to settle for 30 minutes after which the ether fraction was placed in a graduated cylinder and evaporated with N_2 to approximately 40 ml. The water fraction of this primary extraction was extracted a second time with 300 ml of ether in a 2 liter separatory funnel. This extraction was allowed to settle for 16 hours, after which this ether fraction was combined with the first ether fraction (40 ml from first extraction) and both were reduced via N_2 evaporation to 6 ml of concentrated extract. Presumably this was a 50x concentration of the ether soluble lipids from the original blue-green filtrate.

To test the biological activity of the ether fraction; i.e., lipid fraction, 200% and 500% concentrations of the original filtrate level (prior to extraction) were established by adding 0.4 ml and 1.0 ml, respectively, to 10 ml aliquots of B medium. Identifical F and A tests were established in this manner.

Additional controls for the multiple variables in these tests were established as follows:

(A) To verify the existence of the inhibitory activity in the original filtrate, a portion of the filtrate that provided the original 300 ml for the ether extraction was assayed via the usual F vs A assay.

(B) 0.4 ml and 1.0 ml of ether extract were necessary, respectively, for the 200% and 500% concentration tests; therefore, 0.4 ml and 1.0 ml of ether alone were added to B medium to ascertain the effects, if any, of ether-only additions to B medium. Identical F and A tests were established.

(C) 0.4 and 1.0 ml of ether were added to portions of the original filtrate to determine the effects of ether on the original inhibitory activity. This was to explore the possibility that the simple addition of ether might destroy the inhibitor, thus negating all results of the experiment. Identical F and A tests were established.

(D) To determine if any, most, or all, of the inhibitory activity was removed by the extraction, 10 ml portions of the water fraction were placed directly into test tubes. Identical F and A tests were established.

(E) B medium with no additions (excepting the usual ES_I addition) was employed in an F vs A assay to determine the effects of reautoclaving B medium.

Addition of ES_I Metals and of EDTA

To determine if distinctions evidenced in F vs A assays were the result of some unknown effect on the heirarchy of chelation in filtrates, the trace metals included in ES_I (Fe, Mn, Zn, Co) were added to both F and A tests. Metals were added as chlorides at 1, 5, 10, 50, and 10 mg% (by cation weight). The metals in ES_I were chosen for two reasons: first, because they represent metals commonly found to be of significance in the culture of algae; and second, because the addition of ES_I to tests represented a possible causitive factor in the F vs A distinction; *i.e.*, if the chelation capacity of the filtrate was altered during autoclaving, the addition of equal amounts of ES_I to F and A tests may not actually represent the addition of equal amounts of available ES_I metals to F as to A tests.

Similarly, to determine the chelation effects of EDTA, Na_2EDTA was added to 100%, 200%, 300%, and 400% of the ES_I level (calculated at an ES_I addition rate of 1.5%); *i.e.*, Na_2EDTA was added (by weight of molecule) at 9.3, 18.6, 27.9, and 37.2 %, to both F and A tests.

Addition of Vitamins and of Selected Organic Nutrient Sources

To determine whether the capacity of blue-green filtrates to inhibit diatom growth involves the inactivation (binding) of vitamins, a multivitamin (Vitamins, 8A; see Appendix B for complete listing) mix was added to both F and A tests of one filtrate. The vitamin mix was added at 0.2, 0.4, 0.8, 1.6, and 2.0 ml%. This represents vitamin levels in excess of known requirements, or in excess of commonly employed concentrations (0.1 ml%)—when requirements are unknown.

Because an exhaustive survey of the possible organic nutrient sources which might somehow effect the capacity of blue-green filtrates to inhibit diatom growth was impractical, only liver oxoid, yeast extract, and dextrin were considered. The addition levels tested for these three substances were 0.1, 0.5, 1.0, 5.0, and 10.0 mg%. Sterile additions were made to both F and A tests. Comparison and control was established by the usual F vs A assay of the same filtrate.

Although these three compounds are generally considered to contain a variety of organic molecules which might reasonably be used as nutrient sources by auxotrophic, or heterotrophic, algae, these tests in no way exhaust the possible nutrient sources which might be involved in the inhibition. The testing of these complex organics might, however, suggest a limit to the probability that such nutrients are involved.

Non-Linsley Organisms

In order to determine whether the inhibition of diatom growth by heat-labile metabolites of blue-green algae is a phenomenon peculiar to Linsley Pond, or whether it is of more general occurrence in fresh water, non-Linsley algae were selectively employed.

Filtrate Production—two non-Linsley blue-green algae were employed as filtrate producers: Nostoc muscorum, from the Indiana collection and Nostoc sp., from E. Bonanomi's Connecticut River collection. These were grown in the same incubator with the same light, temperature, and media as were Linsley producers; and filtrates of these non-Linsley producers were employed in standard F vs A assays of heat-labile inhibition.

Assay Organisms—non-Linsley diatoms were employed to determine if the inhibition of diatom growth by natural pond waters, and by filtrates of blue-green cultures, was restricted to diatoms from Linsley Pond.

Difficulty with Flagellates—because, as a group, the flagellated forms collected from Linsley are bacterized, axenic non-Linsley flagellates were employed in tests of the sensitivity of flagellates. These tests did not replace tests of Linsley flagellates, but rather, both sets of flagellates were employed in parallel tests.

Effects of Addition of Bacteria to Axenic Producer Cultures

To more accurately determine the effects of bacteria on the heat-labile inhibition of diatom growth by the filtrates of blue-green algae, a 1000 ml producer culture of axenic Anabaena sp. (538) was infected with a 10 ml portion of the bacterial community of the Oscillatoria rubescens (535) culture. O. rubescens (535) was separated from its bacterial community by passing 10 ml of the culture thru a 1.0µ Millipore filter. The strength of the inhibitory capacity of the resulting filtrate was compared with that of a filtrate of the same Anabaena sp. (538) grown axenically.

Results of this comparison permit consideration of the effects of bacteria in four distinct situations:

-bacteria in producer culture, but not in assay culture;
-bacteria in both producer and assay cultures;

-bacteria neither in producer culture, nor in assay culture;
-bacteria not in producer culture, but in assay culture.

This array of comparisons is useful in the interpretation of bacterial effects on inhibitory capacity of filtrates, in general; and in the interpretation of assays of filtrate activity for those producer organisms which were never successfully separated from bacteria, in particular.

Effects of Age of Producer Culture on Inhibitor Strength

To determine if the heat-labile inhibitory substance is released throughout the life of a culture or released mainly at lysis, one half of a filtrate was harvested after one week's growth, the second half was harvested after 15 days growth (lysis). These two halves were then subjected to the F vs A assay and results were compared for differences in inhibitory capacity which would correlate with culture age at harvest.

Effect of Lower Mn Levels in Producer Cultures

To determine if the Mn level in producer cultures was in excess of the optimum level for blue-greens, ES_I was formulated with all Mn omitted from the nutrient complement. Thus only the Mn which occurred naturally in the Linsley water (which was the basic liquid for the B medium in which producer cultures were grown) was present in these producer cultures.

The inhibitory capacity of this filtrate (as demonstrated by the usual F vs A assays) was then compared to that of a producer culture (grown in parallel) which had contained the usual ES_I nutrient addition.

VII:IV PRELIMINARY CHARACTERIZATION OF HEAT-LABILE SUBSTANCE RESPONSIBLE FOR INHIBITION OF DIATOM GROWTH: RESULTS AND DISCUSSION

Introduction

The consistent, heat-labile, inhibition of diatom growth by filtrates of blue-green algae and by Linsley Pond waters was considered of sufficient significance to warrant a detailed study. Chemical, and, or, physical characteristics of the heat-labile molecule postulated to be the basis for this negative activity were sought.

All experiments aimed at the preliminary characterization of the heat-labile inhibitor were done with filtrates of *Anabaena sp.* (538). If only a single assay organism was to be used, *Nitschia frustulum* (224) was chosen. The limit to only one, or a few, assay organisms was essential in these more complex experiments. The multiple controls; *e.g.*, in the ether extraction experiment, would have required prohibitive numbers of replicate tests if a large number of assay organisms had been employed.

Heat-Lability

The heat-labile characteristic of the inhibitor was the first to be studied. It was originally found that the inhibitor was eliminated by autoclaving (15 psi, 120°C) for 20 minutes. Further study indicated that the inhibitor was partially eliminated by 20 minutes at 90°C (atmospheric pressure], but was not measurably diminished by 60°C for 20 minutes. If D.C. medium (Provasoli, McLaughlin, Droop, 1957) was added prior to autoclaving, the inhibitory substance was spared. In preliminary tests of the effects of the inhibitor on marine diatoms the inhibition occurred in both F and A tests if the D.C. was added to the filtrate prior to autoclaving; however, if D.C. was added to the filtrate after autoclaving, then the inhibition in A tests was eliminated. Although studies have not been conducted to determine which of the constituents of the D.C. medium was responsible for the sparing of the inhibitor during autoclaving, other work (concerning the vitamin B_{12} found in Linsley waters) has indicated that Tris buffer alone is sufficient to produce the sparing effect of D.C. for other organic molecules. This suggests that the rise in pH, as dissolved CO_2 is driven out of the water, rather than the high temperature, destroys the inhibitor.

Volatility

The inhibitor is non-volatile. Loosely capped filtrates can be stored for over one month (providing they are kept in darkness at 0-5°C and are axenic) without substantial loss of activity. To further explore the possibility that the inhibitor was a volatile substance N_2 was vigorously bubbled (5 psi) thru an uncapped, freshly-harvested filtrate (150 ml) via a Supreme "very fine" aquarium air stone for 20 minutes. No measurable loss of activity occurred.

Dialysis

The inhibitor is a dialyzable molecule. Dialysis against sucrose was sufficient to isolate the heat-labile inhibitor from the filtrate. This inhibitor can be returned to test tube cultures and will produce F vs A assays similar to those of untreated filtrate. Pore size in the dialysis tubing employed for this work is 50Å, the tubing retains molecules with a mw of 12,000 or more. These results place an upper limit on the size of the inhibitory molecule at 50Å and an upper limit for its mw at 12,000. The high concentration of sugar in these experiments somewhat limited the growth in all tests, both in the dialyzable and non-dialyzable F and A tests.

Separate portions of the dialyzable and non-dialyzable segments of the filtrate were stored for a second assay. One half of each portion was frozen, the other half was stored at 0-5°C. An F vs A assay was run on these separately stored portions approximately six months later. It was found that freezing destroyed the inhibitor while cold storage preserved it. Williasm (personal communication, 1972) has suggested that freezing might initially cause a minor disruption of the structure of the organic molecule (or molecules) responsible for biological activity, but that this form of disruption could be corrected by allowing the frozen sample to rest undisturbed for an unspecified (but relatively long) period. He further speculated that the high concentration of sugar in such samples might mechanically interfere with the recovery of structural integrity of organic molecules in frozen samples.

The growth maxima (expressed as O.D.) of each of the conditions discussed above are provided in Table 9 for illustration and comparison.

Ultrafiltration

Ultrafiltration provided more detailed information concerning the size and character of the inhibitor. Ultrafiltration separated the inhibitor into three distinct molecules:

(1) An heat-labile inhibitor which is retained by the Amicon PM10 Ultrafilter, with a pore size of 20Å and a nominal retention weight based on 90% retention of protein molecules of 10,000.

(2) An heat-labile enhancer which passes thru the 20Å Amicon Ultrafilter (PM10) and which is retained by the 10Å Amicon Ultrafilter (UM2). The nominal retention weight of this filter (based on proteins) is a mw of approximately 1,000. The influence of this enhancer is effectively masked by the inhibitor described above (1).

(3) An heat-labile inhibitor which passes thru both of the previously mentioned ultrafilters and which also passes thru the UM05 ultrafilter with a mw limit of 500 and a pore size of 10Å.

Summary—(1) Heat-labile inhibitor; mw between 1,000 and 10,000; size: 20-50Å. Peach-pink color, oily to touch.

(2) Heat-labile enhancer; mw between 1,000 and 10,000; size: 10-20Å. Vivid mid-yellow color, adheres to filter (suggesting a possible steroid since the UM2 membrane filter is known to bind steroids).

(3) Heat-labile inhibitor; mw between 500 and 0; size: 0-10Å.

The determination of an inhibiting or enhancing quality of these molecules was accomplished by sets of dilution series tests. These indicated the presence of an enhancer by diminished growth with greater dilution, and the presence of an inhibitor by increased growth with greater dilution (Table 10). It should be mentioned that the larger inhibitor (1) is similar in color (peach-pink) and tactile effect (oily) to the inhibitory substance extracted by ether, below. The smaller of the inhibitors (3) has not been characterized to my satisfaction, and may yet prove to be an artifact of the assay system; *i.e.*, since assays were done with diatoms,

TABLE 9 Comparison of Dialyzable and Non-Dialyzable Portions of a Producer Filtrate Before and After Storage. (Growth Maxima expressed as O.D.)

Description of Sample	F	A
Dialyzable, no storage	155	450
Non-Dialyzable, no storage	480	390
Dialyzable, stored at 5°C (6 mos.)	155	400
Non-Dialyzable, stored at 5°C (6 mos.)	375	325
Dialyzable, stored frozen (6 mos.)	410	395
Non-Dialyzable, stored frozen	450	435

1.0 ml sample (as described in text) added to 10 ml test tube portions of B medium

TABLE 10. Results of Dilution Series Tests of Activity of Filtrates after Passage thru Ultrafilters

Percentage Dilution				Heat-Labile Molecular Species Present	Interpretation
0%	33%	66%	100%		
(No filtration)					
180	170	190	380	-1 +2 -3	The combined strength of -1 and -3 even when diluted, cannot be overcome by +2
(Thru PM10 u.f.)					
275	410	385	380	+2 -3	Now that -1 is removed, -3 can be diluted sufficiently so that +2 (33%) can express itself. The 66% dilution is to great for +2 to withstand.
(Thru UM2 and UM05)					
200	215	245	380	-3	Diluting -3 allows increased growth. -3 is sufficiently strong to still be evident at the 33% level.

a small, but additional portion of silica (leached from test tubes during autoclaving, may be present in A tests.

Ether Extraction

The inhibitor can be extracted from the filtrate with ether. This substance is peach-pink color and is oily to the touch. An ether extract, concentrated and added to B medium at approximately 200% of the original level (in freshly harvested 16-day-old filtrates), though not immediately lethal, prevents cell growth and division. A 500% concentration similarly employed causes bleaching and disintegration of the assay inoculum (Table 11). There is an indication that some of the heat-labile inhibition remains in the water fraction after extraction.

Other Nutrient Additions

No pattern of effects was observed as a result of the additions of FeCl, MnCl, ZnCl, CoCl, Vitamins 8A (see Appendix B for listing), liver oxoid, yeast extract, or dextrin. Results with Na_2EDTA additions require additional study for interpretation. Higher levels appear to improve growth in both F and A tests, but there is no pattern presently clear as to an effect which would eliminate inhibition.

Possible Generalization of Inhibition of Diatoms by Heat-Labile Metabolites of Blue-Green Algae

As can be seen from results summarized in Table 5, filtrates of the two non-Linsley blue-greens tested evidenced inhibitory, or neutral, effects on the growth of Linsley diatoms. While the limit of testing to only two non-Linsley blue-greens makes generalization tenuous at best, there are two points which must be considered: first, these tests suggest a likely generalization of diatom inhibition by blue-greens (tests of non-Linsley diatoms also suggest this); and second, though the distribution of negative and neutral results in Table 5 may not make it self-evident, close observations of these tests indicate a much more obvious inhibition when producer and assay organisms are from the same body of water. This obvious, and common, form of inhibition might not have been recognized if original test organisms had been from various collections (therefore, from various bodies of water). This point gives great significance to the choice of experimental organisms. When ecologically significant principles are sought, it is most desirable that experimental materials including, especially, organisms be from the same ecosystem. Morphologically (taxonomically) identical organisms may be very dissimilar in their physiological requirements.

Non-Linsley assay diatoms included LP and Hillsdale, pinnate diatoms, from central New York State (L. Provasoli isolates); a *Fragilaria crotenensis* from Fuller Pond in northwestern Connecticut (K. Glaus-Porter isolate); a mixed Fragilariaceae culture and a *Tabularia sp*. from the North Brunswick Reservoir, Connecticut (J. Lehman isolates); *Navicula pellicu-*

TABLE 11 Comparison of Control and Test Culture Maxima in Ether Extraction Procedures

Description of Test	F	A
Untreated filtrate	190	300
Untreated filtrate with 0.4 ml ether added	220	285
Filtrate after ether extraction	275	350
B Medium*	455	470
B Medium with 1.0 ml ether added	410	520
B Medium with 0.4 ml ether added	420	470
B Medium with 1.0 ml ether and 500% concentration of Inhibitor	0	540
B Medium with 0.4 ml ether and 200% concentration of Inhibitor	0	510

*Comparisons of growth in test cultures must be made with growth in the B Medium control. Tests were run in B Medium with extract, or other, additions. B Medium has a higher concentration of nutrients than does the filtrate. Filtrates were tested to insure that this filtrate would exhibit the basic inhibition. To date all filtrates have exhibited the inhibition; however, its presence cannot be assumed.

losa (from the Indiana collection), and *Skeletonema costatum* and *Thalassiosira* from L. Provasoli's collection. All evidenced an inhibition of growth by filtrates of Linsley blue-greens. A minimal testing of the marine diatoms suggests that this inhibition also effects marine forms; however, the ecological significance of the inhibition in freshwater systems cannot be readily generalized to marine systems because of 1) the obvious dilution possibilities in ocean waters; 2) the minimum occurrence of blue-green blooms in marine environments, and 3) the myriad of differences in the chemical effects of fresh and marine waters. Nevertheless, a study of *Tricodesmium* blooms might prove interesting at some future date.

Effects of Bacteria on Diatom Inhibition

Some of the producer cultures and some of the assay cultures used in this study were bacterized. This bacterization did not totally eliminate the inhibition of diatom growth produced by filtrates of blue-green algae, but it did appear that the presence of bacteria in either the producer or the assay culture lessened the potency of the inhibitor and, further, it appeared that the presence of bacteria in both the producer and the assay culture had an even greater effect on the inhibition. To determine if the presence of bacteria (presumably some of the bacteria would be able to metabolize the inhibitory molecules) did actually lessen inhibition an axenic culture of *Anabaena sp.* (538) was infected with a 10 ml aliquot of bacterized (algae-free) filtrate from one of the *Oscillatoria rubescens* (535) cultures. This newly bacterized culture was then used as the inoculum for a 1000 ml producer culture. The comparison of the inhibitory capacity of this bacterized producer of *Anabaena sp.* (538) with the inhibitory capacity of an axenic producer culture of *Anabaena sp.* (538) indicated that the original premise was correct. That is, a comparison of "bacterized" F tests with axenic F tests indicates greater growth (less inhibition) in the "bacterized" group. In general the "bacterized" A tests while they did support greater growth than "bacterized" F tests, did not achieve the maxima of axenic A tests. This may reflect the competitive use of some essential nutrients, such as PO_4, by the bacteria in the bacterized producer cultures. Table 12 provides a comparison of results obtained from this work.

Mn Effect

Although the subject requires considerable additional study, it is worthy of note that the higher Mn level in producer cultures enriched by 1½% ES_I, as compared to producer cultures enriched by ES_I which was formulated without Mn, apparently lessened the level of inhibition exhibited by filtrates of these cultures. This suggests a stronger competitive position for blue-greens in waters with low natural stores of Mn—reminiscent of the findings (under very different circumstances) of R. Patrick (1969).

TABLE 12. Comparison of Inhibitory Effect of Bacterized and Axenic Producer Cultures of *Anabaena* sp. (538)

Assay Organism	Bacterized		Axenic	
	F	A	F	A
Nitschia frustulum (224)	240	370	180	380
Synedra famnilica (202)	180	120	90	120
Synedra sp. (299)	105	115	80	120
Fragilaria sp. (99)	70	75	65	80
Cyclotella sp. (211)	190	215	65	170
Nitschia sp. (352)	130	370	130	245
Tabellaria sp. (764)	80	100	110	140
Asterionella formosa (800)	115	125	110	140

VII:V EFFECTS ON DIATOM INHIBITION OF SILICA ADDITION TO FILTRATES OF BLUE-GREEN ALGAE AND TO FREEZER-STORED AND FRESHLY-COLLECTED LINSLEY POND WATERS

Methods

To determine the effects of silica addition on the diatom inhibition evidenced in F vs A assays, silica—as Na_2SiO_3—was added to assays of both blue-green filtrates and natural pond waters. Initially, additions were established at 20 mg% (by weight of hydrated molecule). Later 1, 5, 10, and 20 mg% additions were used.

The second set of addition levels was employed in tests designed not only to determine effects of Si addition on assays, but also to determine the availability of silica in the natural waters of the pond at the times of collection.

Results and Discussion

A suggestion by L. Margulis (personal communication, 1973) that the blue-green algae might effect an inhibition of diatom growth by binding silica, making it less available to diatoms even at times when silica levels were apparently sufficient, prompted the study of silicate addition as a means of overcoming the heat-labile inhibitor. $Na_2SiO_3 \cdot 9H_2O$ was added to filtrates and to pond waters, and F vs A assays were established.

Silica addition did improve growth in most cases, but this improvement often occurred in both F and A assays with the result that there was still considerably less diatom growth in F than in A cultures (Table 13). These results were promising, however, since the addition of other substances in a variety of attempts to overcome inhibition produced no effects. Additionally, in some cases the added silica did appear to eliminate the inhibition.

Some organisms responded negatively to high Si addition levels and this response occurred at lower levels of silica addition in A tests than it did in F tests. This disparity suggests an initially greater available Si pool in A tests than in F tests. Since the absolute silica level in both F and A tests must be very similar (autoclaving of A tests could not account for the leaching of as much as 20 mg% of silica from test tubes), the possibility of a heat-labile silica binder, or inactivator, must be considered. Further work on this point is needed to clarify its significance and to determine whether this silica effect is a part, or the whole, of the inhibitory effect which can be studied via ether extraction, ultrafiltration, or dialysis (see discussions above).

The annual cycle of silica levels in Linsley, as reported by Cowgill (1970), do not suggest that simple silica limitation can be the basis for the absence of diatom blooms in the pond. Laboratory tests, however, indicate that silica is a determining factor in the ending of those spring

TABLE 13 .The Effects of Silica Addition on the Heat-Labile Diatom Inhibition Exhibited by Filtrates of Blue-Green Algae. (20mg% added)

PRODUCER *Anabaena* sp. (538)					
	With Silica Addition		Without Silica Addition		
Assay Organism	F	A	F	A	Interpretation
Nitzschia frustulum (224)	420	270	220	420	420 is the max. growth for this culture. Too little, or too much silica limits.
Synedra sp. (299)	180	180	75	105	180 is the max. growth for this culture. A levels are inadequate Si addition does not exceed tolerance of assay organism.
Fragilaria sp. (99)	125	125	75	90	125 is the max. growth for this culture. A levels are inadequate Si addition does not exceed tolerance of assay organism.
Cyclotella sp. (211)	0	330	0	140	There is a different kind of inhibition here. (It is heat-labile toxicity)

PRODUCER *Synechecoccus* sp. (91)					
	With Silica Addition		Without Silica Addition		
Assay Organism	F	A	F	A	Interpretation
Synedra famnilica (202)	20	0	50	0	A non-heat-labile inhibition is present. Silica addition has no effect. Growth is possible if filtrate is charcoal treated. (Max. = 170)
Fragilaria sp. (99)	130	170	80	115	Silica addition has no effect on inhibition. Generally increases growth.
Nitzschia frustulum (224)	300	155	245	330	300-330 is the max. growth for this cult. Too little or too much Si limits growth.

diatom blooms which do occur. Water collected immediately after the end of the spring diatom bloom in 1973 was tested for its ability to support diatom growth. The addition of silica (as $Na_2SiO_3 \cdot 9H_2O$ at 20 mg%) tripled the growth of most of the diatoms tested (Table 14). The addition of increasing amounts of silica to Linsley water collected prior to the 1974 diatom bloom did produce increasingly positive effects on the growth of assay diatoms. The same additions to water collected at the end of the same, 1974, bloom also produced significant improvements in growth; however, changes were notably greater in post bloom waters. Figures 23 thru 26 illustrate this distinction between pre- and post-diatom bloom waters for the spring of 1974.

As demonstrated in Figure 23 when filter sterilized water was tested, maximum growth (approximately O.D. 350) for pre-bloom water was achieved by the addition of 5 mg% sodium silicate. This same growth maxima was achieved for post-bloom filtered water by the addition of more than 20 mg%. When autoclaved water samples are compared (Figure 24) maximum growth in pre-bloom waters is achieved with an addition of only 1 mg% sodium silicate while more than 10 mg% is required for post-bloom maxima.

Both situations indicate a much greater silica deficit in post-, then in pre-bloom waters; however, if autoclaved and filtered samples from the same date are considered, an additional informative contrast can be observed. In pre-bloom waters (Figure 25) A samples required only 1 mg% to achieve growth maxima between 300 and 400, but F samples required 5 mg%. Similarly in post-bloom waters (Figure 26) F samples required 20 mg% addition to achieve growth maxima of O.D. 300, while autoclaved samples required only 10 mg%. Suggesting that some of the silica in the water samples for either date was not available to the diatoms until after the water samples were autoclaved. Circumstantial evidence only that some of the heat-labile diatom inhibition exhibited during this study is related to silica availability.

TABLE 14 Diatom Growth Maxima in Pond Water Collected at the End of the 1973 Diatom Bloom (May, 13, 1973) With and Without Silica Addition
(20 mg% added)

Organism	With Silica Addition		Without Silica Addition	
	F	A	F	A
Nitschia frustulum (224)	315	315	145	335
Synedra famnilica (202)	120	170	70	120
Synedra sp. (299)	140	190	45	85
Fragilaria sp. (99)	125	155	30	95
Cyclotella sp. (211)	230	280	60	160
Nitschia sp. (352)	210	230	70	245
Tabellaria sp. (764)	160	205	55	65
Asterionella formosa (800)	185	205	60	115

FIGURE 23. COMPARISON OF GROWTH RESPONSE TO VARIOUS LEVELS OF ADDED SILICA IN PRE- AND POST DIATOM-BLOOM POND WATERS.
FEBRUARY 14, AND JUNE 13, 1974
FILTER-STERILIZED ONLY.

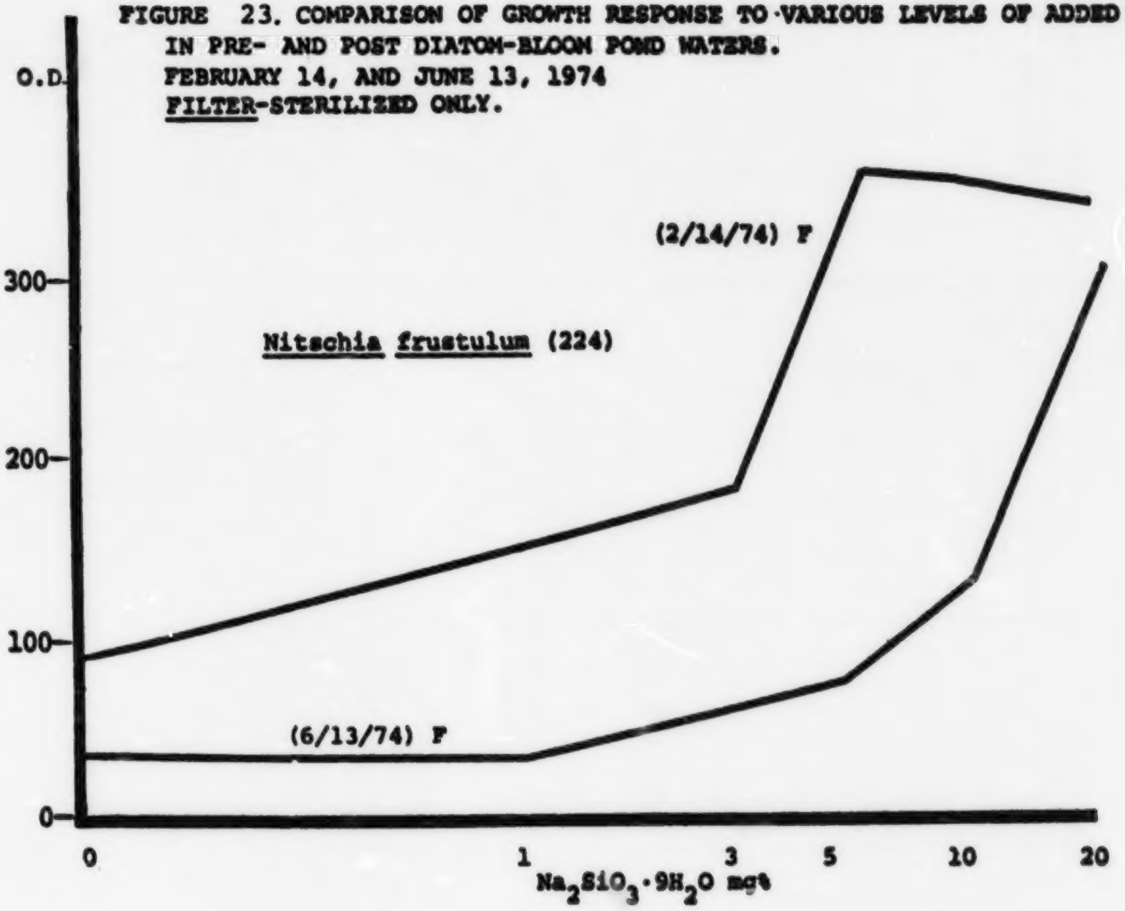


FIGURE 24 COMPARISON OF GROWTH RESPONSE TO VARIOUS LEVELS OF ADDED SILICA IN PRE- AND POST DIATOM-BLOOM POND WATERS. FEBRUARY 14, AND JUNE 13, 1974 AUTOCLAVED.

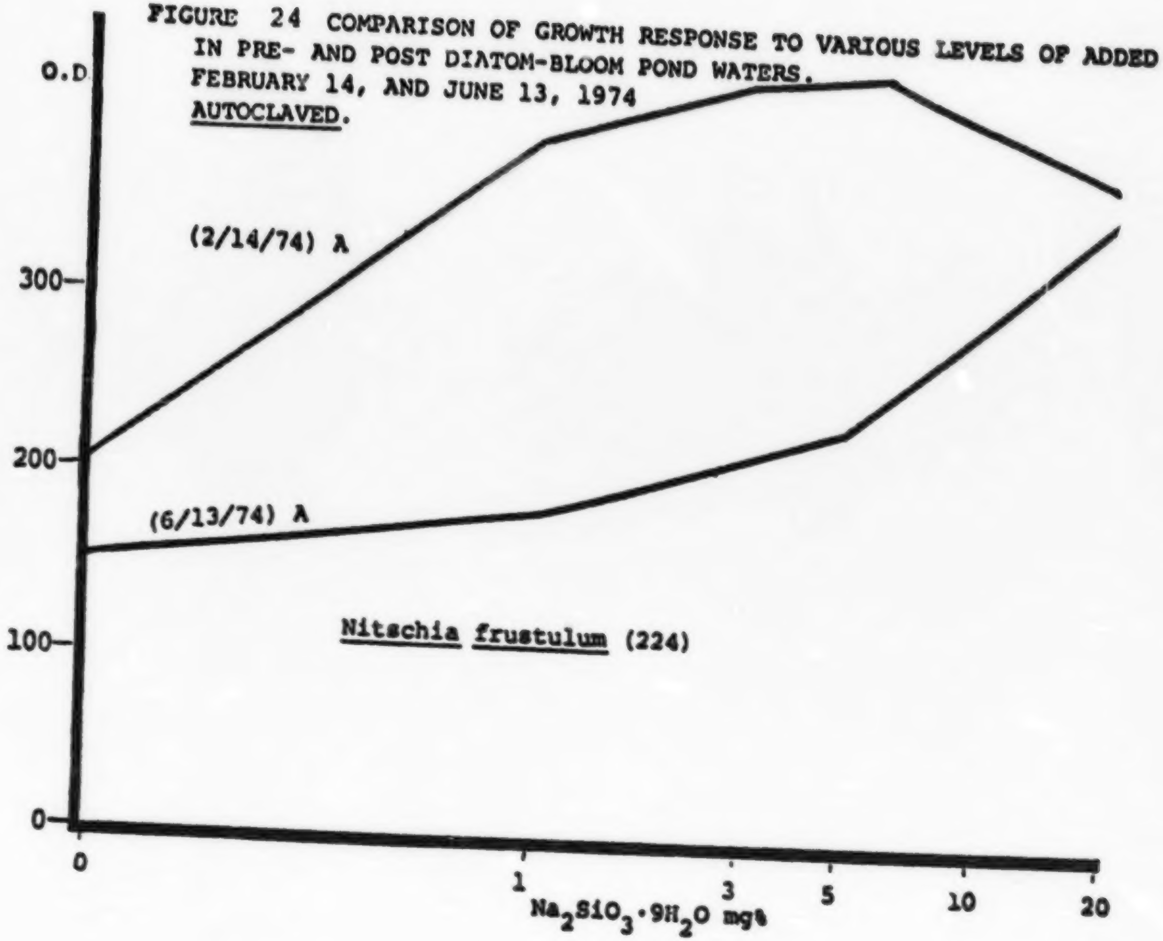


FIGURE 25 COMPARISON OF GROWTH RESPONSE TO VARIOUS LEVELS OF ADDED SILICA PRE-DIATOM-BLOOM POND WATERS.

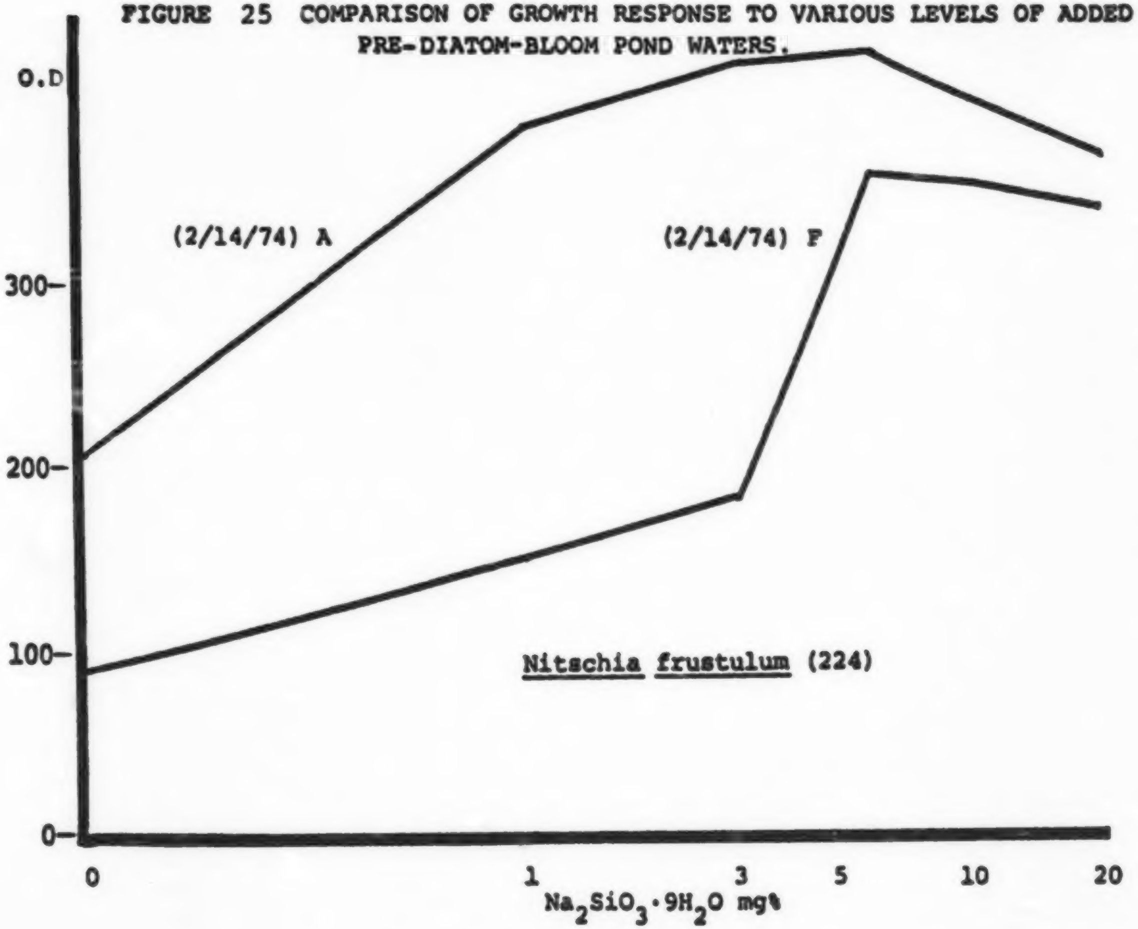
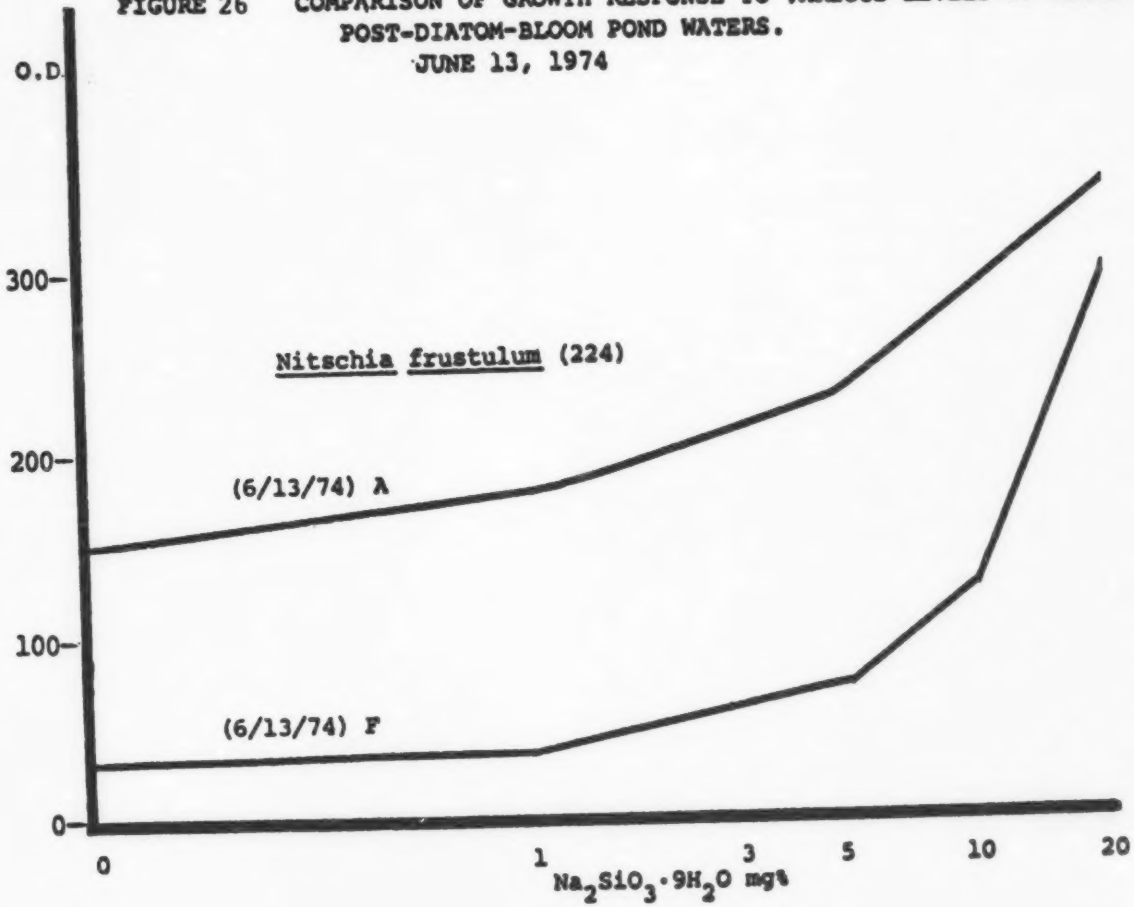


FIGURE 26 COMPARISON OF GROWTH RESPONSE TO VARIOUS LEVELS OF ADDED SILICA POST-DIATOM-BLOOM POND WATERS. JUNE 13, 1974



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APPENDIX A

SOME PREVIOUSLY REPORTED BLOOMS IN LINSLEY POND

Linsley Pond has been considered moderately eutrophic, or mesotrophic, for at least four decades. Many occurrences of "blooms" have been reported by other investigators. Often such reports are simply descriptive additions which were incidental to the main interest of a given paper, but which were included for background, or interpretative, value. A more thorough understanding of the historic development of conditions of eutrophication in Linsley Pond will be available in the near future when R. Brugam completes his study of cores taken from Linsley in conjunction with his dissertation research at Yale.

Listed below are some of the reports of blooms in Linsley.

Organism	Descriptive Terms Employed	Date of Bloom	Source of Information
Synedra	5140/liter	Approx. 1938 growing season	Riley, 1938
Tabellaria	2170/liter	"	"
Fragilaria	640/liter	"	"
Anabaena, Microcystis, Coelosphaerium	extensive blooms	August & Sept. 1936	Riley, 1939
Dinophyceae and Chrysophyceae	occasionally great blooms	September 1937 to June 1938	Riley, 1939
Fragilaria	dominant form in blooms	September 1937	"
Anabaena and Coelosphaerium	most important in blooms	August 1937	"
Oscillatoria	dominant genus	most of winter and early spring 1937-1938	"
peridinians, Cyclotella, Mallomonas Eudorina	great phytoplankton burst-seeded from inflow water	January 1938	"

APPENDIX A

SOME PREVIOUSLY REPORTED BLOOMS IN LINSLEY POND (cont'd.)

Organism	Descriptive Terms Employed	Date of Bloom	Source of Information
Oscillatoria, *Synedra*, *Melosira*, *Scenedesmus*, peridinians	spring phytoplankton burst	April 1938 surface, and thru June in deeper waters	Riley, 1939
Dinobryon, *Fragilaria*, *Asterionella*	sparser bloom than above	May-June 1938 surface	"
Small *Oscillatoria*, larger peridinians	Bloom	Mid-winter	"
(Clorophyceae)	(never dominant)	1937-1938	"
Anabaena	large amount	August 22, 1937	Hutchinson, 1941b
Anabaena circinalis	water-bloom	June 25, 1938	"
Anabaena	Bloom	July 25-August 5, 1938	"
Oscillatoria	large population	September 1941-1942	Hutchinson, 1943
Fragilaria crotonensis, *Synedra acus*, *Asterionella formosa*	abundant	general statement	"
Diatoms	dominated	April 1937	Hutchinson, 1944
Oscillatoria and other Myxophyceae	vernal maxima	other years than 1938-1941	"
Dinobryon	dominant	May-June 1938-1941	"

APPENDIX A
SOME PREVIOUSLY REPORTED BLOOMS IN LINSLEY POND (cont'd.)

Organism	Descriptive Terms Employed	Date of Bloom	Source of Information
Fragilaria crotonensis and *Anabaena circinalis*	enormous development	July 1937-1941 late summer 1938-1941	Hutchinson, 1944
Oscillatoria	relatively large	autumnal 1938-1941	"
blue-green	considerable population	winter 1938-1941	"
Scenedesmus sp. and *Asterionella formosa*	transitory developments	winter 1938-1941	"
Oscillatoria	rather monotonous	1942	"
Synedra acus	immense population	April 17, 1937	"
Fragilaria crotonensis	enormous multiplication	end July 1937 August 3	"
Anabaena circinalis	enormous multiplication	August 10, 1937	"
Oscillatoria prolifica and *O. rileyi*	bulk of plankton bloom	Spring 1938 (March)	"
Dinobryon	(graph maxima)	May-June 1938	"
Aphanizomenon	spring maxima	Spring 1939	"
Dinobryon	maxima after blue-greens or diatoms	March, April, and June 1939	"
Oscillatoria and *Aphanizomenon*	immense population in deeper water (6 m)	July 1944	Hutchinson, 1946
Oscillatoria prolifica	Bloom	April 1945	"
Oscillatoria prolifica	main autumnal maxima	November 1945	"
Anabaena and *Oscillatoria*	dominant but poor growth	October 14, 1952	Benoit, 1957

APPENDIX B

MEDIA USED DURING THIS STUDY

The following media were used with some frequency during this study. Other media were tested but those listed were found to be of greatest value.

ES_I - enrichment	(Provasoli, 1968)
pH 7.8	
$NaNO_3$	350 mg%
Na_2 glycerophosphate	50 mg%
FeEDTA	2.5 mg%
P II metals	25 ml
Vitamin B_{12}	10
Thiamine	0.5 mg%
Biotin	5γ
Cu (as Cl)	25γ
Tris	500 mg%
H_2O	to 100 ml

ES_{Isi} - enrichment	(Provasoli, unpublished)
$Na_2SiO_3 \cdot 9H_2O$	150 mg% added to ES_I

Use 2 ml of stock per 100 ml natural water

FeEDTA (Provasoli, 1968)

351 mg $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$

330 mg Na_2EDTA

H_2O to 500 ml

P II Metals	(Provasoli, 1968)
Na_2EDTA	100 mg%
Fe (EDTA)	1 mg%
Boron	20 mg%
Mn (as Cl)	4 mg%
Zn (as Cl)	0.5 mg%
Co (as Cl)	0.1 mg%
H_2O	to 100 ml

FW	(Provasoli, unpublished)
pH 7.0-7.5	
*K_2 glycerophosphate	0.63 mg%
$Ca(NO_3)_2 \cdot 4H_2O$	4.2 mg%
Ca_3 citrate$\cdot 7H_2O$	4.3 mg%
$MgSO_4 \cdot 7H_2O$	0.7 mg%
$MgCl_2 \cdot 6H_2O$	1.7 mg%
FeEDTA	0.1 mg%
Mn (as Cl)	0.001 mg%
Zn (as Cl)	0.03 mg%
ethyl silicate	3.3 mg%
Na_2CO_3	1.0 mg%
H_2O	to 100 ml

*substituted Na for K (sometimes)

DC (Provasoli, McLaughlin, and Droop, 1957)

pH 7.8-8.0

NaCl	1.8%
KCl	60 mg%
$NaNO_3$	50 mg%
$MgSO_4 \cdot 7H_2O$	0.5%
Ca (as Cl)	10 mg%
Tris	0.1%
K_2HPO_4	3 mg%
$Na_2SiO_3 \cdot 9H_2O$	20 mg%
P II metals	3 ml/100
FeEDTA	0.01 mg%
Thiamine	10 γ %
Biotin	0.1 γ %
B_{12}	0.3 γ %
NaH glutamate	50 mg%
Glycine	50 mg%
Na lactate	50 mg%
Sucrose	50 mg%
Na acetate	50 mg%
H_2O	to 100 ml

Cy II (Provasoli, unpublished)

pH 7.6

NH_4 acetate (CH_3COONH_4)	20 mg%
P II metals	1 ml/100
FeEDTA	0.04 mg%
Ca (as Cl)	1 mg%
KCl	3 mg%
$MgSO_4 \cdot 7H_2O$	10 mg%
glycerophosphoric acid	5 mg%
B_{12}	0.1 γ%
S_3 vitamins	0.5 ml/100
Tris (Sigma 121)	33.3 mg%
H_2O	to 100 ml

S_3 Vitamins (Provasoli, McLaughlin, and Droop, 1957)

Thiamine	5 mg%
Biotin	10 γ%
PABA	100 γ%
Folic acid	20 γ%
Nicotinic acid	1 mg%
Thymine	30 mg%
Inositol	50 mg%
Ca panthotenate	1 mg%
H_2O	to 100 ml

DA (D'Agostino and Provasoli, 1970)

pH 6.5

K_2HPO_4	2 mg%
Na_3 citrate	2 mg%
$MgSO_4 \cdot 7H_2O$	2 mg%
Fe (EDTA)	0.2 mg%
Thiotone	60 mg% (Baltimore Biological Labs)
Trypticase	16 mg%
Yeast extract	5 mg%
H_2O	to 100 ml
Agar	25% for semi-solid

CV7 (Provasoli, unpublished)

pH 7.6

Ca (as Cl)	2 mg%
$NaNO_3$	10 mg%
$MgSO_4 \cdot 7H_2O$	4 mg%
Na_2 glycerophosphate	5 mg%
KCl	5 mg%
B_{12}	0.01γ %
Biotin	0.01γ %
Glycylglycine	50 mg%
P IV metals	1 ml/100
H_2O	to 100 ml

CV_7 (thiamine)	(Provasoli, unpublished)
CV_7 + thiamine	10γ%

P IV Metals	(Provasoli and Pintner, 1960)
Na_2EDTA	100 mg%
FeEDTA	4 mg%
Mn (as Cl)	1 mg%
Zn (as Cl)	0.5 mg%
Co (as Cl)	0.1 mg%
Mo	0.5 mg%
H_2O	to 100 ml

Ger 8/10	(Provasoli, unpublished)
Ca (as NO_3)	0.96 mg%
K_2HPO_4	1.0 mg%
$MgSO_4 \cdot 7H_2O$	2.5 mg%
Na_2CO_3	2.0 mg%
$Na_2SiO_3 \cdot 9H_2O$	4.4 mg%
FeEDTA	0.056 mg%
Citric acid	0.39 mg%
H_2O	to 100 ml

TA (Provasoli, unpublished)

pH 7.2

Trypticase	0.1%
Thiotone	0.1%
KCl	0.01%
$MgSO_4 \cdot 7H_2O$	0.01%
Na acetate	0.05%
KH_2PO_4	0.005%
Thiamine	0.05 mg%
H_2O	to 100 ml

FD II (Provasoli, unpublished)

pH 6.5

Ca_3 citrate	5 mg%
NH_4NO_3	2 mg%
$MgSO_4 \cdot 7H_2O$	2.5 mg%
Na_2 glycerophosphate	1.0 mg%
Fe EDTA	0.07 mg%
Vitamins 8A	0.2 ml/100
VT metals	0.1 ml/100
H_2O	to 100 ml

VT Metals	(Provasoli, unpublished)
Zn	10 mg%
Mn	4 mg%
Co	0.3 mg%
Cu	0.03 mg%
V	0.1 mg%
Ti	0.3 mg%
Cr	0.01 mg%
Ni	0.01 mg%
Sr	30 mg%
Mo	0.3 mg%

The following broad range vitamin addition was sometimes added with ES_I when new isolates were sought.

Vitamins 8A	(Tatewaki and Provasoli, 1963)
Thiamine HCl	20 mg%
Biotin	50 γ%
B_{12}	5 γ%
Folic acid	0.25 mg%
PABA	1 mg%
Nicotine acid	10 mg%
Thymine	80 mg%
Chlorine H_2 citrate	50 mg%
Inositol	100 mg%
Putrescine 2HCl	4 mg%
Riboflavin	0.5 mg%
Pyridoxamine 2HCl	2 mg%
Orotic acid	26 mg%
Folinic acid	20 γ%
Ca panthotenate	10 mg%
Pyridoxine 2H	4 mg%
H_2O	to 100 ml

The following trace metal additions were at times added with ES_I when new isolates were sought.

Guillard Metals (Guillard, 1974)

1.0 ml% of this stock = Guillard's recommendations.

Na_2 EDTA 43.6 mg% (of Na_2 EDTA)

	(amt. of element)	(amt. of compound)
$FeCl_2 \cdot 6H_2O$	Fe 6.5 mg%	31.5 mg%
$CuSO_4 \cdot 5H_2O$	Cu 25 γ%	0.1 mg%
$ZnSO_4 \cdot 7H_2O$	Zn 50 γ%	0.22 mg%
$CoCl_4 \cdot 6H_2O$	Co 25 γ%	0.1 mg%
$MnCl_2 \cdot 4H_2O$	Mn 0.5 γ%	1.8 mg%
$Na_2MoO_4 \cdot 2H_2O$	Mo 25 γ%	0.06 mg%
H_3BO_3	B 1.7 mg%	10.0 mg%
H_2O	to 100 ml	

U II Metals (Aldrich and Wilson, 1960)

0.3 ml of this stock = Provasoli's recommendation.

Na_2EDTA	15 mg%
$FeCl_2 \cdot 4H_2O$	0.1 mg%
$CuCl_2$	0.1 mg%
$MnCl_2 \cdot 4H_2O$	1.0 mg%
$ZnCl_2$	0.5 mg%
$NiCl_2 \cdot 6H_2O$	0.1 mg%
$AlCl_3 \cdot 6H_2O$	0.5 mg%
$CoCl_2 \cdot 6H_2O$	0.5 mg%
$RbCl$	1.0 mg%
$BaCl_2$	0.1 mg%
TiO_2	0.5 mg%
$ZrOCl_3 \cdot 8H_2O$	0.5 mg%
$K_2Cr_2O_7$	0.5 mg%
$SrCl_2 \cdot 6H_2O$	0.5 mg%
$Na_2MO_4 \cdot 2H_2O$	0.5 mg%
H_3BO_3	0.5 mg%
$CsCl$	0.5 mg%
$(NH_4)_2Ce(NO_3)$	0.1 mg%
$CdCl_2 \cdot 2\frac{1}{2}H_2O$	0.1 mg%
$SnCl_2 \cdot 2H_2O$	0.1 mg%
$RuCl_3$	0.1 mg%
$RhCl_3$	0.1 mg%

APPENDIX C

LISTING OF SPECIFIC EFFECTS OF FILTRATES ON INDIVIDUAL ORGANISMS

PRODUCER: *Oscillatoria rubescens* (535) - bacterized

EFFECT	ASSAY ORGANISM	EFFECT	ASSAY ORGANISM
0	Oscillatoria rubescens (535)b*	0	Nitschia frustulum (224)a
0	O. rubescens (739)b	-	Nitschia sp. (352)a
+	Oscillatoria sp. (776)a	-	Asterionella formosa (800)b
+	Pseudanabaena galeata (597)a	-	Synedra sp. (299)a
+	Anabaena sp. (538)a	0	Synedra famnilica (202)a
+	Anabaena sp. (765)b	0	Fragilaria sp. (99)b
+	Aphanizomenon flos-aquae (766)b	-	Tabellaria sp. (764)b
0	Synechecoccus sp. (91)a	-	Cyclotella (211)b
+	Ankistrodesmus convolutus (105)a	+	Trachelmonas sp. (778)a
-	Scenedesmus quadricauda (314)a	0	Synura uvella (43)b
+	Staurastrum sp. (207)a	-	Chlamydomonas sp. (298)b
		+	C. reinhardtii (+)a non-Linsley
		+	C. reinhardtii (-)a non-Linsley
		0	C. reinhardtii (+ & -)a non-Linsley
		+	Euglena gracilis-a non-Linsley
		0	monad (317)b
		+	Wolnscynskia limnetica-a non-Linsley
		+	Dinobryon cylindricum-b non-Linsley

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*An a or b following culture identification means assay culture was either axenic or bacterized.

PRODUCER: Oscillatoria rubescens (739) - bacterized

EFFECT	ASSAY ORGANISM
0	Oscillatoria rubescens (535)b
-	O. rubescens (739)b
0	Oscillatoria sp. (776)a
+	Pseudanabaena galeata (597)a
+	Anabaena sp. (538)a
-	Anabaena sp. (765)b
+	Aphanizomenon flos-aquae (766)b
-	Synechecoccus sp. (91)a

EFFECT	ASSAY ORGANISM
-	Nitschia frustulum (224)a
-	Nitschia sp. (352)a
-	Asterionella formosa (800)b
-	Synedra sp. (299)a
-	Fragilaria sp. (99)b
-	Tabellaria sp. (764)b

EFFECT	ASSAY ORGANISM
-	Ankistrodesmus convolutus (105)a
+	Scenedesmus quadricauda (314)a
+	Staurastrum sp. (207)a

EFFECT	ASSAY ORGANISM
0	Trachelmonas sp. (778)a
0	Synura uvella (43)b
0	Chlamydomonas sp. (298)b
0	C. reinhardtii (+)a non-Linsley
-	C. reinhardtii (-)a non-Linsley
+	C. reinhardtii (+ & -)a non-Linsley
0	Euglena gracilis-a non-Linsley
0	monad (317)b
+	Wolnscynskia limnetica-a non-Linsley
+	Dinobryon cylindricum-b non-Linsley

PRODUCER: Oscillatoria sp. (776) - axenic

EFFECT	ASSAY ORGANISM	EFFECT	ASSAY ORGANISM
-	Oscillatoria rubescens (535)b	-	Nitschia frustulum (224)a
-	O. rubescens (739)b	-	Nitschia sp. (352)a
0	Oscillatoria sp. (776)a	-	Asterionella formosa (800)b
-	Pseudanabaena galeata (597)a	0	Synedra sp. (299)a
-	Anabaena sp. (538)a	-	Synedra famnilica (202)a
0	Anabaena sp. (765)b	-	Fragilaria crotenensis non-Linsley
-	Aphanizomenon flos-aquae (766)b		
-	Synechecoccus sp. (91)a		

-	Kirchneriella obesa (104)a	-	Eudorina sp. (767)b
-	Ankistrodesmus convolutus (105)a	+	Sphaerellopsis sp. (598)b
-	Scenedesmus quadricauda (314)a	-	Chlamydomonas sp. (298)b
-	Staurastrum sp. (207)a	0	C. reinhardtii (+ & -)a non-Linsley
-	Closterium sp. (499)a	0	Euglena gracilis-a non-Linsley
0	chlorococcal green algae (307)a	0	monad (317)b

PRODUCER: Pseudanabaena galeata (597) - axenic

EFFECT	ASSAY ORGANISM	EFFECT	ASSAY ORGANISM
0	Oscillatoria rubescens (535)b	-	Nitschia frustulum (224)a
0	O. rubescens (739)b	-	Nitschia sp. (352)a
0	Oscillatoria sp. (776)a	0	Asterionella formosa (800)b
-	Pseudanabaena galeata (597)a	-	Synedra sp. (299)a
-	Anabaena sp. (538)a	-	Synedra famnilica (202)a
-	Anabaena sp. (765)b	-	Fragilaria sp. (99)b
-	Aphanizomencn flos-aquae (766)b	-	Cyclotella (211)a
+	Synechecoccus sp. (91)a	-	Fragilaria crotenensis non-Linsley
+	Kirchneriella obesa (104)a	0	Sphaerellopsis sp. (598)b
+	Ankistrodesmus convolutus (105)a	0	Chlamydomonas sp. (298)b
+	Scenedesmus quadricauda (314)a	-	C. reinhardtii (+ & -)a non-Linsley
+	Staurastrum sp. (207)a	-	monad (317)b
+	Closterium sp. (499)a		
+	chlorococcal green algae (307)a		

PRODUCER: Anabaena sp. (538) - axenic

EFFECT	ASSAY ORGANISM	EFFECT	ASSAY ORGANISM
+	Oscillatoria rubescens (535)b	-	Nitschia frustulum (224)a
-	O. rubescens (739)b	-	Nitschia sp. (352)a
-	Oscillatoria sp. (776)a	-	Asterionella formosa (800)b
0	Pseudanabaena galeata (597)a	-	Synedra sp. (299)a
0	Anabaena sp. (538)a	-	Synedra fammilica (202)a
-	Anabaena sp. (765)b	-	Fragilaria sp. (99)b
+	Aphanizomenon flos-aquae (766)b	-	Tabellaria sp. (764)b
+	Synechecoccus sp. (91)a	-	Cyclotella (211)a
-	Kirchneriella obesa (104)a	-	Fragilaria crotenensis non-Linsley
0	Ankistrodesmus convolutus (105)a		
0	Scenedesmus quadricauda (314)a		
+	Staurastrum sp. (188)a		
0	Staurastrum sp. (200)a		

PRODUCER: Anabaena sp. (762) -bacterized

EFFECT	ASSAY ORGANISM	EFFECT	ASSAY ORGANISM
-	Oscillatoria rubescens (535)b	-	Nitschia frustulum (224)a
-	O. rubescens (739)b	-	Asterionella formosa (800)b
-	Oscillatoria sp. (776)a	+	Synedra sp. (299)a
-	Pseudanabaena galeata (597)a		
+	Anabaena sp. (538)a		
-	Anabaena sp. (765)b		
+	Aphanizomenon flos-aquae (766)b		
+	Synechecoccus sp. (91)a		

PRODUCER: Aphanizomenon flos-aquae (766) - bacterized

EFFECT	ASSAY ORGANISM	EFFECT	ASSAY ORGANISM
+	Oscillatoria rubescens (535)b	-	Nitschia frustulum (224)a
0	O. rubescens (739)b	-	Nitschia sp. (352)a
+	Oscillatoria sp. (776)a	0	Asterionella formosa (800)b
0	Pseudanabaena galeata (597)a	0	Synedra sp. (299)a
+	Anabaena sp. (538)a	0	Fragilaria sp. (99)b
0	Anabaena sp. (765)b	-	Tabellaria sp. (764)b
-	Aphanizomenon flos-aquae (766)b	-	Cyclotella (211)a
0	Synechecoccus sp. (91)a		

EFFECT	ASSAY ORGANISM	EFFECT	ASSAY ORGANISM
0	Kirchneriella obesa (104)a	+	Synura uvella (43)b
+	Ankistrodesmus convolutus (105)a	0	Chlamydomonas sp. (298)b
0	Scenedesmus quadricauda (314)a	0	C. reinhardtii (+ & -)a non-Linsley
0	Staurastrum sp. (185)a	0	Euglena gracilis-a non-Linsley
+	Closterium sp. (499)a	0	monad (317)b
+	chlorococcal green algae (307)a		

PRODUCER: Synechecoccus sp. (91) - axenic

EFFECT	ASSAY ORGANISM
-	Oscillatoria rubescens (535)b
-	O. rubescens (739)b
+	Oscillatoria sp. (776)a
-	Pseudanabaena galeata (597)a
-	Anabaena sp. (538)a
+	Anabaena sp. (765)b
+	Aphanizomenon flos-aquae (766)b
+	Synechecoccus sp. (91)a

EFFECT	ASSAY ORGANISM
-	Nitschia frustulum (224)a
-	Nitschia sp. (352)a
-	Asterionella formosa (800)b
-	Synedra sp. (299)a
-	Synedra famnilica (202)a
-	Fragilaria sp. (99)b
-	Tabellaria sp. (764)b
-	Cyclotella (211)a
-	Fragilaria crotonensis non-Linsley

-	Kirchneriella obesa (104)a
+	Ankistrodesmus convolutus (105)a
-	Scenedesmus quadricauda (314)a
0	Staurastrum sp. (188)a
0	Staurastrum sp. (200)a

-	Sphaerellopsis sp. (598)b
-	Chlamydomonas sp. (298)b
+	C. reinhardtii (+ & -)a non-Linsley
+	Euglena gracilis-a non-Linsley
+	monad (317)b

APPENDIX D

THE O.D. METER EMPLOYED DURING THIS STUDY

The optical density meter built by Dr. Fred Kavanaugh for Dr. Provasoli functions in a manner similar to that of the Spec-20 with a test tube insert module; i.e., it accepts test tubes and does not require decanting of cultures into cuvettes. As mentioned in the text, this permitted close surveillance of culture growth throughout the lifespan of a culture.

Density units were read from 0.000 thru 0.999 and expressed as reading x 1000 to eliminate the necessity for a decimal. Repeatability was excellent, consistent readings were obtained to the nearest thousandth on all occasions. It was necessary to mark each test tube to insure that it was not rotated for different reading occasions. This was the result of test tubes being considerably less consistent in terms of thickness and pregrowth transparency than would be cuvettes which were made especially for spectrometer reading. A mark was placed on each test tube prior to inoculation at the position in which a zero reading could be obtained. Actually, even if this precaution were not to be taken, the ordinary test tubes were remarkably consistent, varying at maximum from 0.000 to about 0.025 when rotated in the light beam.

To provide assurance as to the consistent performance of this meter (which is unfamiliar apparatus to all except those who have spent time in Dr. Provasoli's laboratory), its readings are compared on the following page to those of a Beckman Spectrometer set at 425.

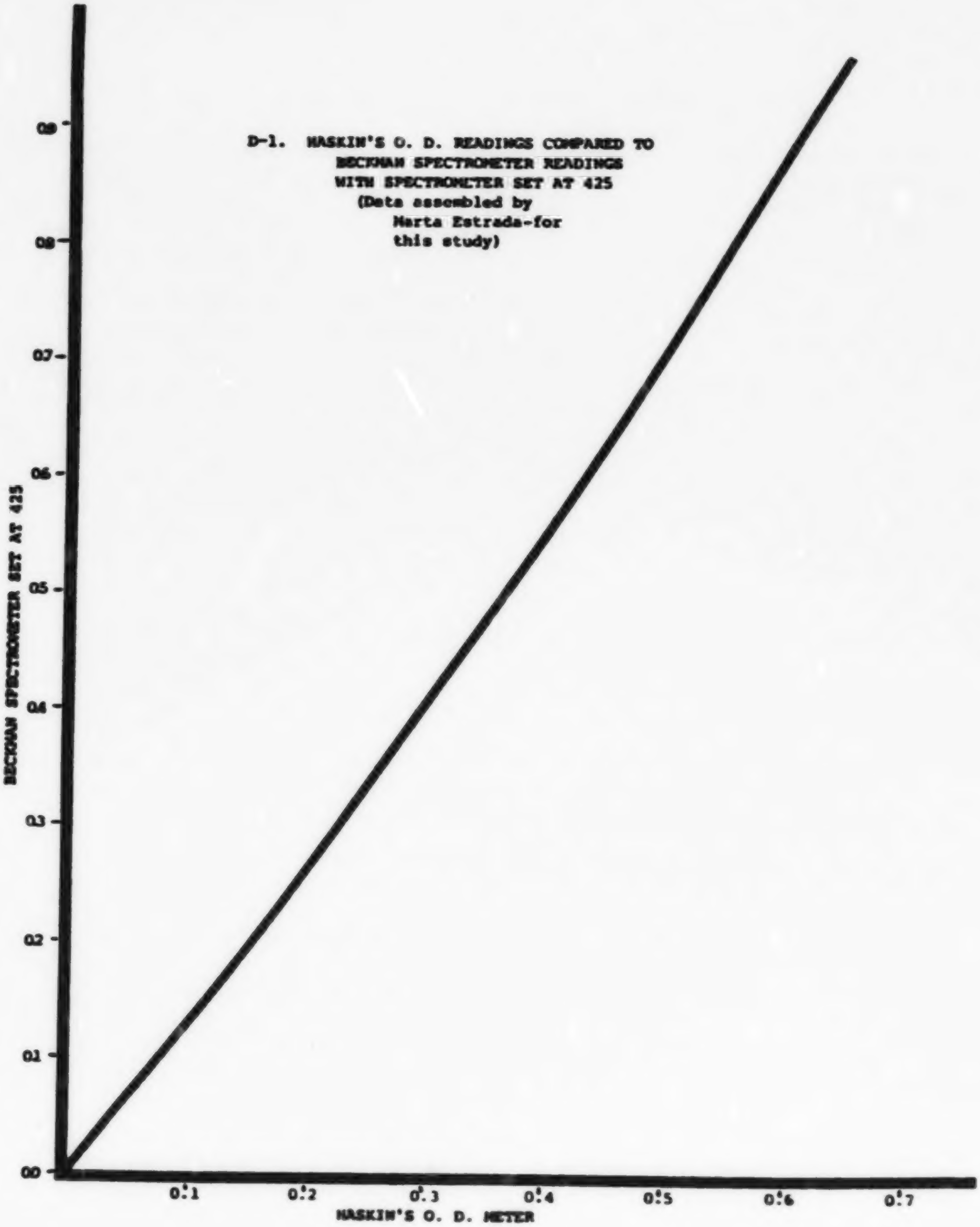
To indicate the consistent relationship of O.D. readings to cell counts the following pages contain translation graphs based on organisms commonly employed for experimental purposes.

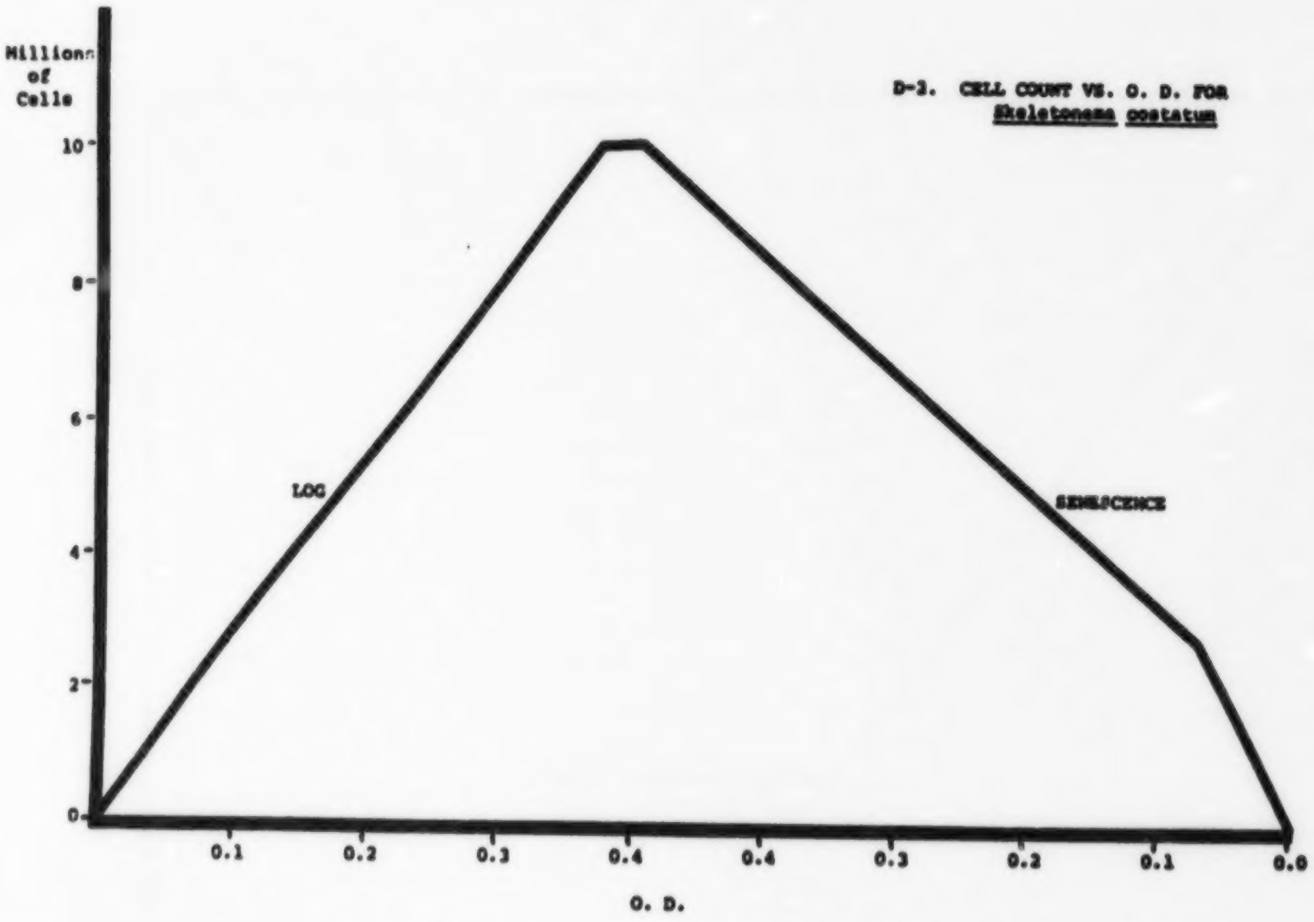
D-1. HASKINS O.D. READINGS COMPARED TO BECKMAN SPECTROMETER READINGS WITH SPECTROMETER SET AT 425.

D-2. CELL COUNT VS O.D. FOR SKELETONEMA COSTATUM

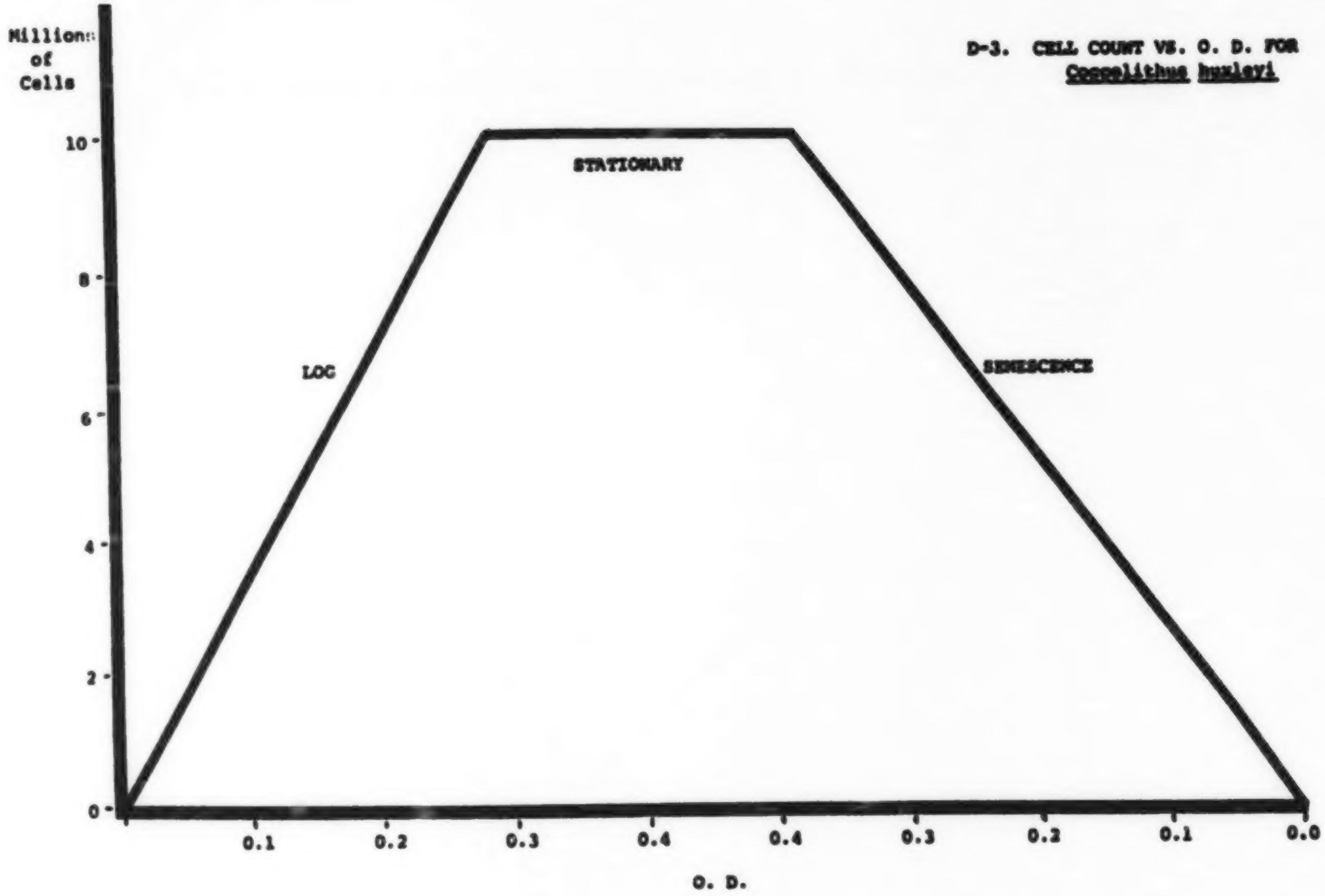
D-3. CELL COUNT VS O.D. FOR COCCOLITHUS HUXLEYI

D-4. CELL COUNT VS O.D. FOR NITSCHIA FRUSTULUM v. INDICA SKVORTZOW

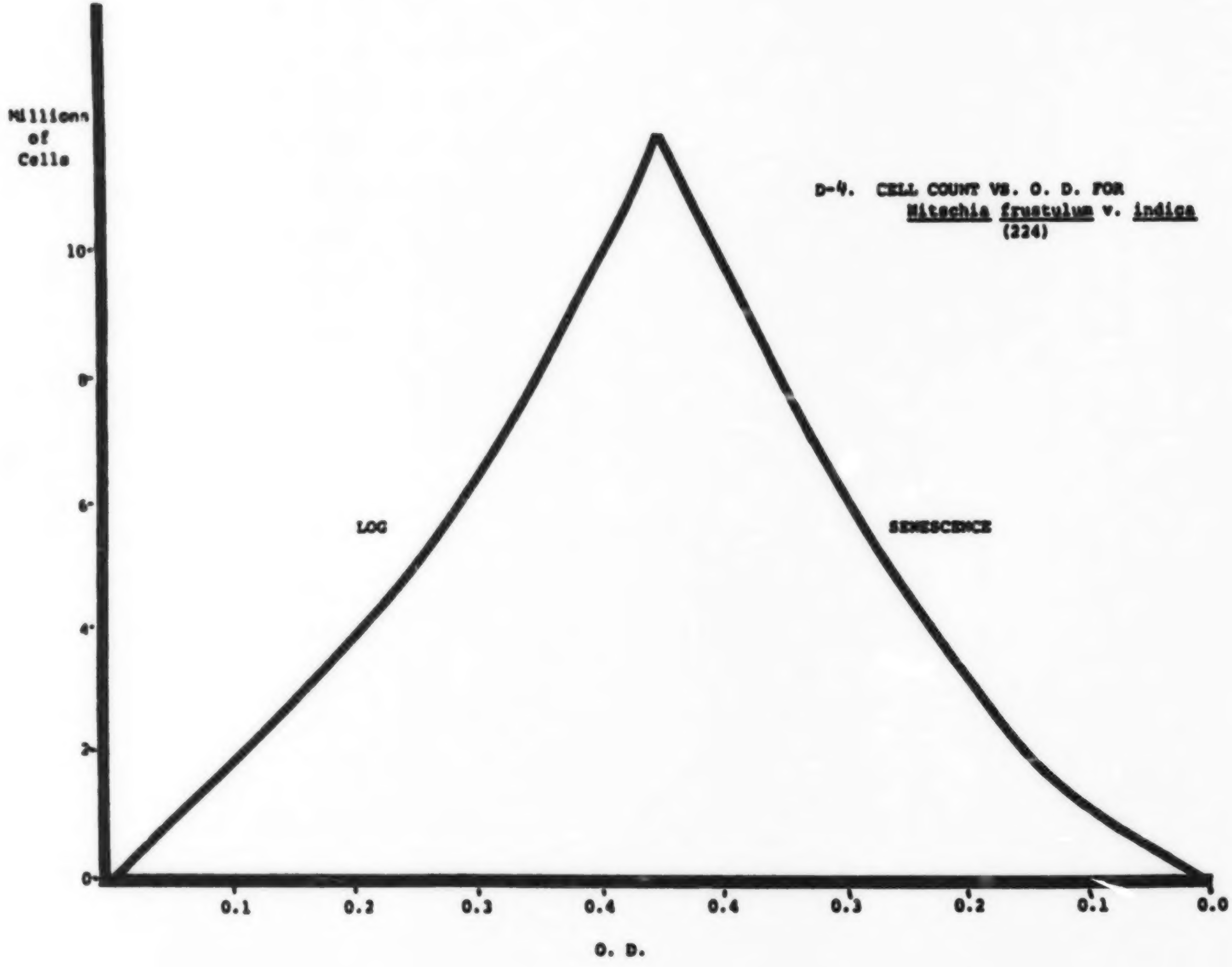

D-1. HASKIN'S O. D. READINGS COMPARED TO BECKMAN SPECTROMETER READINGS WITH SPECTROMETER SET AT 425 (Data assembled by Marta Estrada-for this study)



D-2. CELL COUNT VS. O. D. FOR
Skeletonema costatum



D-3. CELL COUNT VS. O. D. FOR *Coccolithus huxleyi*



D-4. CELL COUNT VS. O. D. FOR
Nitschia frustulum v. *indica*
(224)